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STUDIES ON BUD DORMANCY OF WOODY SPECIES

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ABSTRACT

The role of endogenous inhibitors and growth substances in the regulation of bud dormancy of wood plants was investigated using mature trees, seedlings, isolated shoots, and aseptically cultured buds and shoots.

The photoperiodic induction of dormancy was not mediated through changes in inhibitor β activity, or ABA levels of buds and leaves of *Alnus viridis* seedlings. Changes in the activity of the inhibitor β fraction of buds and leaves of field grown *Alnus glutinosa* occurred during autumn but were not correlated with the onset of dormancy.

Exogenous applications of 10^{-4}M ABA, 10^{-3}M CCC, ABA + CCC, 10^{-3}M C5, 10^{-3}M C9 and 10^{-3}M C10 to actively growing *Alnus viridis* seedlings did not result in the formation of dormant buds. Dormancy was not induced by pruning the root system of actively growing alder seedlings or by the treatment of isolated growing shoots with 10^{-4}M ABA.

ABA prolonged the dormancy of apparently dormant buds after their transfer to an environment favouring growth, but this effect was modified by species, time of year, presence and absence of leaves, and position of the bud on the stem. C8 and C9 prolonged the dormancy of *Populus nigra* Italica while C10 promoted bud burst in *Salix alba/babylonica*. CCC was ineffective in most species tested (*Alnus*, *Populus* and *Salix*) except when in combination with ABA.

The use of aseptic shoots of *Populus yunnanensis* cultured *in vitro* as a bioassay for dormancy-inducing substances was found to be limited by the variable growth responses elicited. No dormancy-inducing substances were detected in extracts, including the inhibitor β fraction, of dormant buds or leaves or shoots. The presence of various concentrations of ABA, C5, C10, AMO 1618 and ABA + AMO 1618 in the medium did not cause the formation of dormant shoot morphology, although growth was inhibited by certain treatments.

Results are discussed in relation to the inhibitor hypothesis of dormancy regulation.

CHAPTER 1

INTRODUCTION

Shoot growth in temperate woody species is characterised by a cyclic pattern of activity and inactivity. This pattern of activity, alternating with inactivity or bud dormancy, is closely correlated with the change in seasons and the associated climatic conditions. In spring, as temperatures rise and daylength increases, bud burst and shoot growth commences and continues into summer as long as environmental conditions are favourable. Shoot growth, which is a consequence of cell divisions occurring at the bud meristem and the subsequent elongation of these cells, results in an overall increase in tree height. Although a wide range of patterns in shoot growth among tree species exists, four basic patterns are generally recognised. Where the overwintering bud contains all the organ primordia, preformed in an embryonic state, and the extension growth and maturation of this preformed shoot are accomplished in the spring, the shoot growth is termed single flush. This determinate pattern of shoot growth is exemplified by such species as blue spruce (*Picea pungens*), horse-chestnut (*Aesculus hippocastanum* L.), mountain beech (*Nothofagus solandri* var. *solandri*), and ponderosa pine (*Pinus ponderosa*). Following the realisation of the current season's elongation potential, the initiation and establishment of the next growing season's elongation potential, in primordial form, takes place within the newly developed buds during the summer. In single flush species, growth is restricted to a single period early on in the growing season and little or no elongation or increase in height occurs after this. In contrast to this a second type of shoot growth, recurrent shoot growth, occurs in some *Quercus* and *Pinus* species, where there are two or more distinct "waves" or flushes of extension growth separated by periods of inactivity during the course of the growing season. Each flush ends with the formation of a new terminal bud. A third type of shoot growth, termed continuous growth, occurs in those tree species where the preformed telescoped shoot, which is contained in the overwintering bud, undergoes extension and maturation in the spring, and is immediately followed by further uninterrupted extension growth, including the initiation and development of new foliage leaves. This pattern of shoot growth is

found in several species, including *Betula, Populus, Acer* and *Alnus*. These trees are less determinate in their growth habit, and continue to grow in height and form new leaves throughout the growing season until late summer-early autumn. The fourth type of shoot growth is typified by the Y shaped or sympodial growth habit as seen in elm (*Ulmus*) species. Following a short period of extension growth and maturation of the preformed embryonic shoot, the terminal bud aborts or undergoes transition into a reproductive structure. Where the apical bud is regularly aborted, the axillary bud immediately below the aborted apex commences growth and this results in the shoot axis being Y shaped.

These four patterns of shoot growth are maintained during the spring and summer months until late summer and early autumn when decreasing daylength and lower night temperatures cause the bud meristem activity to slow and eventually cease. At this stage the bud undergoes a change in development, such that there is an intensive preparation for both the overwintering bud state and the new spring growth. In some single flush species, the preparation for the next spring's growth may already be complete. The change in development of the bud ensures its survival during the winter months.

Changes occur in the morphology, anatomy, metabolism, and function of the bud meristem. The most visible change is the production of a short succession of scales instead of the normal series of enclosing young leaves around the meristem and leaf primordia. The leaf primordia themselves may develop into the much heavier bud scales or, as found in cottonwood (*Populus deltoides*), leaf primordia development is suppressed and the bud scales develop from the stipules. The bud scales remain tightly appressed around the bud and by their close fitting shape and much thicker, resistant texture, give the inner tissues protection from freezing injury. Once an adequate or necessary number of scales have been formed, the meristem continues to undergo cell division and morphogenesis to form a succession of vegetative leaf or flower primordia. As stem elongation declines, these primordia remain in this embryonic state as a miniature telescoped shoot over the winter period. In single flush species, the entire complement of next season's leaves will be preformed at this stage.

Some species may not produce bud scales, but use the young underdeveloped leaves to afford protection during the winter period.

In other species, there is only a gradual change in morphology from young leaf to bud scale and back to leaf. However, in all cases, survival of the bud during the period of low winter temperatures results.

Many biochemical and physiological changes also occur during the change in bud development. These changes are reflected in the ability of the bud to tolerate or survive low temperatures, including freezing temperatures. In many cases this tolerance may be a direct result of the physical presence of the bud scales themselves, but in cases where the bud scales are only slight modifications of the true leaf form, the nature of the tolerance is within the bud itself. To some extent, all buds of temperate woody plants, irrespective of the nature of their bud scales, undergo biochemical and physiological changes to avoid cold injury to the susceptile meristematic tissue.

As daylength decreases and the temperatures decline, with the onset of winter, the bud completes its intense preparation and enters a period of low metabolic activity. The bud at this stage is termed dormant or at rest, and will remain in this state during most of the winter. It should be noted that the cessation of active growth, the formation of the next season's leaves, and the morphological and biochemical changes that lead to the acquisition of cold tolerance, occur well before there is any danger of frost. In some species this may be completed in summer when conditions are still favourable for growth.

During winter, activity within the bud does not cease completely and slow differentiation of internal organs may still continue, except in the very coldest weather. For most woody species, exposure of the overwintering bud to a minimum period at low temperatures (approximately $5^{o}C$) is essential for bud burst in the spring. The total length of this winter chilling may be used by the bud as a measure of time to indicate winter is past. At the end of winter chilling, the bud begins to swell and the close fitting bud scales become looser. With daylength increasing progressively and daily temperatures rising, the once dormant bud is now in a state which enables the preformed telescoped shoot to undergo rapid extension as soon as environmental conditions are favourable.

Bud burst occurs in the spring and with the subsequent extension and maturation of the embryonic shoot, the seasonal cycle of activity

and inactivity is complete. It should be noted that many deviations from this general description of the growth and dormancy cycle exist. Nevertheless, most temperate woody species show a seasonal growth cycle which enables them to survive the temperate climate.

The tree is able to co-ordinate growth and development to match the seasonal climatic changes. Growth occurs during the time when conditions are most favourable, that is, during the warm temperatures and long photoperiods of spring and summer. By undergoing changes in bud development, and entry into the dormant state well before the onset of winter, the tree is able to survive the adverse conditions of winter. Furthermore, the tree has evolved a means of being able to cope with the much shorter fluctuations or sudden changes in environmental conditions. For example, abnormally low and high temperatures during summer and winter result in the arrest of bud growth until conditions again become favourable. The cyclic pattern of shoot growth and its correlation with seasonal climatic conditions provides an excellent means by which woody species can survive the environmental conditions they experience in their temperate habitats.

What, then, are the environmental signals that are responsible for this cycle of growth and development in woody species. The major climatic factors affecting the growth cycle of the shoot are photoperiod, temperature, water and nutrient availability. Of these, photoperiod appears to be the key agent in controlling the cessation of growth and the development of dormancy (Wareing, 1956). However, photoperiodic control of the development of dormancy can be by-passed or overridden by temperature, light intensity, availability of nutrients and soil moisture as well as certain shock treatments. Furthermore, those environmental conditions inducing dormancy are different from those that are responsible for the emergence from dormancy. Before discussing these factors in more detail, it is necessary first to define dormancy and the dormant state, and to introduce some terms.

1.1 DORMANCY AND THE DORMANT STATE

An accurate definition of dormancy is difficult to achieve. This is because there is a gradual transition between the phases of active growth and inactivity or dormancy, and the existence of an

overlap between the various developmental phenomena associated with growth and dormancy. Dormancy is not merely the cessation of growth and metabolism, but a developmental phase in its own right. Whilst all division and stem elongation slows down in actively growing buds towards the end of summer, there is in fact, at this time, an increase in the rate of cell division and morphogenesis of new leaf primordia and bud scales. The bud also undergoes various biochemical and physiological changes. Therefore, there is a need for a definition of dormancy to include morphological symptoms and physiological changes that occur during dormancy development.

The classical concept of dormancy is based on the ability of an organ to elongate or grow in some other manner. Doorenbos (1953) used the term "dormancy" in its widest sense to apply to "any case in which a tissue predisposed to elongate does not do so." Hence, in common usage and especially in the ecological sense, the term "dormancy" meant a temporary suspension of visible growth and development without any reference to causal factors. However, the existence of various phases or physiological states in the development of dormant buds necessitates a more specific definition of dormancy.

At least three phases or levels of dormancy development can be recognised (Samish, 1954; Romberger, 1963). These are (1) development leading to the dormant state, (2) the dormant state, and (3) release from the dormant state leading to the non-dormant or quiescent state. Unfortunately, several different terms have been used by different authors to differentiate between the various phases in the development of dormancy. In addition, other authors have recognised these phases of dormancy as being distinctly different types of dormancy and have introduced other specific terms (Romberger, 1963). This has further confused and complicated the definition of dormancy. The nomenclature used in this study follows that of Wareing and Saunders (1971) and Saunders (1978a).

In the first phase, dormancy development, there is a progression from growth cessation through to the winter bud formation, which may or or may not have occurred under the influence of correlative inhibition. This period of imperceptible growth marks the beginning of dormancy. At this stage the buds are not intrinsically dormant, but are prevented from elongating by some stimulus arising elsewhere in the plant.

For example, some terminal buds and most axillary buds have their growth inhibited by the activities of other organs such as mature leaves. The buds are said to be in a state of correlative dormancy during this phase. Other terms such as summer dormancy (Doorenbos, 1953), early rest (Perry, 1971), and pre-dormancy (Wareing, 1969) have also been used to describe this dormancy phase. At first, active growth may be artificially renewed by treatments such as defoliation, excess nitrogen fertilization and exposure to long photoperiods. However, under natural conditions of late summer (shortening photoperiods and low night temperatures) it becomes increasingly more difficult to renew growth until finally, the treatment has no effect and the bud is then said to have reached a state of innate dormancy. This is the second phase of dormancy; the dormant state or innate dormancy and is defined as a state in which, even though normally favourable conditions of warm temperatures, adequate nutrition, water and aeration are supplied, growth and development do not take place until a separate set of conditions (winter chilling) has been experienced. At this stage, bud burst is delayed as a result of conditions arising within the bud itself. The duration of the innate dormant state is extremely variable (Romberger, 1963). In some species it may not exist at all, whereas in others, the buds remain in this state until they have been exposed to a period of cold temperatures or chilling during the winter. Temperatures near $5^{o}C$ are most effective and those temperatures above or below this optimum result in a longer chilling period. The state of innate dormancy has also been termed deep dormancy, true dormancy (Wareing, 1969), winter dormancy (Doorenbos, 1953) and rest (Samish 1954, Romberger, 1963). In this literature review and study, these terms are considered synonomous and the term dormancy is used in this restricted sense.

The third phase of dormancy involves the gradual release or emergence from the innate dormancy phase. Once the buds are no longer innately dormant they are capable of renewed extension growth in the later part of winter or early spring. However, no visible growth occurs until the environmental conditions are favourable. Usually, a minimum time of exposure to warm temperatures is necessary. The buds are said to be in a phase of dormancy variously referred to as quiescence (Romberger, 1963), post-dormancy (Wareing, 1969), imposed dormancy (Doorenbos, 1953), and after rest (Perry, 1971). This phase or

quiescence is defined as the prevention of growth by the absence of one of the basic conditions for normal growth, e.g. low temperatures, insufficient water. This phase of dormancy is imposed by the external environment and growth resumes as soon as the inhibiting conditions are removed.

The phases of dormancy are not mutually exclusive and may overlap in time. They serve only to help plant physiologists describe dormancy as a phenomenon, which involves a gradual transition from the reversible state of imperceptible growth, to the state of innate dormancy and through to the state in which the plant is capable of renewed shoot growth. That different phases or states of bud dormancy exist does not appear to be in dispute, but there appears to be no agreement on the number and nature of the different phases or states of dormancy which may exist [e.g. see Romberger (1963), Smith and Kefford (1964), Giertych (1974), De Maggio and Freeberg (1969), Tyurina (1979), Holbo, Askren and Hermann (1981)]. Most workers accept the concept of correlative or summer dormancy, innate dormancy, and quiescence or imposed dormancy and consider innate dormancy to be a more intense expression of correlative dormancy with the same underlying cause. Others consider correlative dormancy and innate dormancy to be distinctly different physiological events or states. More detailed physiological studies are required to resolve this disagreement and to develop a more satisfactory system of nomenclature and defintion of dormancy.

1.2 ENVIRONMENTAL FACTORS CONTROLLING THE INDUCTION OF DORMANCY

Among the factors which greatly influence growth activity are temperature, photoperiod, quality of light, nutrition, and water supply. The growth processes of woody plants are usually a result of the combined effect of these factors. However, of the environmental factors experienced by temperate zone tree species, daylength or photoperiod is the most important factor controlling the duration and cessation of extension growth and the time of onset of dormancy (Downs and Borthwick, 1956a,b; Wareing, 1956; Nitsch, 1957a,b; Kramer and Kozlowski, 1960; Vince-Prue, 1975). In general, the rate and duration of extension growth are increased by long days, whereas under short days, extension

growth is decreased and the onset of dormancy hastened. Four major types of responses to photoperiod are recognised amongst woody species (Vince-Prue, 1975). The first includes species, such as black locust (*Robinia pseudoacacia*), birch (*Betula pubescens*), red maple (*Acer rubrum*), and European larch (*Larix decidua*). These species grow indefinitely under long days (LD's), as long as temperatures are favourable, and cease growth under short days (SD's). For these species, there appears to be a critical daylength above which growth occurs and below which dormancy is induced. In the second type of response, growth is not maintained indefinitely under LD's and the onset of dormancy is delayed but not prevented, as seen in sycamore (*Acer pseudoplatanus*), horse chestnut (*Aesculus hippocastanum*), sweet gum (*Liquidamber stryraciflua*). The third type of response is seen in those species in which extension growth proceeds in a series of flushes. Here, the duration of the period between successive flushes is decreased by LD's and in some cases growth may appear to be continuous. This type of photoperiod response is seen in *Pinus sylvestris*, and in some *Citrus* species. Finally, there are species which are insensitive to daylength; these include species of cultivated fruit trees such as *Pyrus*, *Malus* and *Prunus*.

There appears to be no exceptions to the rule, that in species where the onset of dormancy is a response to photoperiod, dormancy is accelerated by SD's and delayed or prevented by LD's. However, the responses of woody species to photoperiod have been mostly studied in seedlings because of their advantage over mature trees in such experimentation. Nevertheless, there is some evidence to suggest photoperiod is also important in the induction of dormancy in mature trees growing under natural conditions. Kramer (1936) was able to show that dormancy is induced in mature black locust (*Robina pseudoacacia*) trees when photoperiod falls to 12 hours, but with exposure to additional light, growth will continue until temperatures fall. Similarly, other evidence (Wareing, 1956; Alvim, Thomas and Saunders, 1978) indicates, that in species where growth continues well into autumn and is responsive to SD's, photoperiod controls the onset of dormancy, e.g. in *Populus*, *Betula*, *Larix* and *Salix* species. However, extension growth in some species ceases in mid summer, well before days begin to shorten appreciably, and it seems that endogenous conditions appear to override photoperiod effects, even though experimentally,

many of these species show a response to daylength (Wareing, 1956). Furthermore, as noted by Wareing (1948), mature trees seem to have a much shorter period of extension growth than seedlings of the same species. Wareing (1969) suggests that internal conditions, such as competition between the various shoots and branches for nutrients and organic metabolites within the tree itself, determine the period of growth and the onset of dormancy. However, those buds that are formed under LD's are not innately dormant, but in a state of correlative or summer dormancy since defoliation usually causes such buds to grow out. It is not until later on in the growing season, when daylength is declining rapidly, that the buds became innately dormant. Hence, entry into dormancy is still a short day response, although the formation of the dormant morphology occurs under LD's. Similarly, the formation of axillary buds occurs under LD's, but they are held in check by correlative inhibition and SD's are required for dormancy induction, as shown in birch (Wareing and Black, 1958) and blackcurrant (Nasr and Wareing, 1961).

Further evidence of the influence of photoperiod on growth cessation and dormancy development in mature trees is obtained from studies on species showing a wide geographic distribution. Such species show marked ecotypic differences in photoperiodic responses in relation to latitude and altitude at which they occur naturally (Vaartaja, 1959; Vince-Prue, 1975). Races growing near the equator require shorter photoperiods for active extension growth than do the more northern races adapted to the longer natural photoperiods (Wareing, 1956; Heide, 1974; Juntilla, 1976, 1980; Downs and Bevington, 1981). Similarly the studies of Habjørg (1972), Perry and Hellmers (1973), and Heide (1974) revealed that the critical daylength for ecotypes of *Betula pubescens*, *Betula verrucosa*, *Salix caprea*, *Alnus incana*, *Acer rubrum* and *Picea abies* increased with increasing latitude and altitude. Such studies indicate that woody species become well adapted to the natural daylength conditions experienced in their specific habitats, and that daylength is responsible for controlling their seasonal cycle of growth and dormancy.

Daylength not only influences the onset of dormancy but also other dormancy related phenomena, such as cambial activity, leaf fall, and chilling or cold resistance in some species (Wareing, 1956; Weiser, 1970; Vince-Prue, 1975). However, in the case of leaf fall and chilling

resistance, temperature is also of importance and under natural conditions an interaction between temperature and daylength is thought to control the development of these processes (Perry, 1971; Vince-Prue, 1975).

The features of the photoperiodic control of dormancy have been described and are similar to those recognised in flowering (Vince-Prue, 1975). The site of perception for the induction of dormancy by SD's seems to be the youngest, just fully expanded or partly expanded leaves (Wareing, 1954; Waxman, 1957; Nitsch, 1959). These tissues are most sensitive, but in birch the much younger, unexpanded leaves in the apical region also show a high degree of sensitivity to photoperiod (Wareing, 1954). Since the dormancy response occurs at the shoot apices, at least one transmissible stimulus must be involved. This was demonstrated (Wareing, 1954) in *Acer pseudoplatanus* and *Robinia pseudoacacia* by exposing the shoot apex and mature leaves of the same plant to different photoperiods. Both these species require the mature leaves to be exposed to SD's for the shoot apex to become dormant. In birch, however, it is the bud itself which responds to daylength, and therefore, a transmissible stimulus may not be necessary. On the other hand, *Quercus robur* requires both the apex and leaves to be exposed to LD's in order to maintain growth. Therefore, the bud may be just as important as mature leaves in some species. In this case the transmissible stimulus could be from the young, unexpanded foliage leaves and leaf primordia to the meristem region of the apex.

As found for flowering, a critical daylength for the induction of dormancy is recognised in those species where growth is maintained indefinitely under LD's. However, as in flowering, it is the duration of the daily dark period or night length, rather than the photoperiod, which is important. If the dark period is interrupted by a short light break near the middle of the dark period, then dormancy is delayed or prevented (Wareing, 1950; Zahner, 1955). Nitsch and Somogyi (1958, cited in Vince-Prue, 1975), showed that a night break of 30 to 60 minutes was sufficient to prevent the induction of dormancy by SD's in several species.

The wavelength sensitivity of the night break response has been studied only in a few cases (Nitsch, 1957a, 1963), and there has been no recorded attempt of an action spectrum for dormancy effects. The problem is that induction of dormancy is a slow process and quantitative

assessments are not simple. However, experiments have demonstrated that red light is most effective in preventing dormancy by light-break treatment given during the dark period in several species (Phillips, 1941; Nitsch, 1957a, 1962, 1963). This suggests that phytochrome is involved in dormancy induction as in flowering. However, red/far-red reversibility in relation to dormancy has been demonstrated only in a few cases, and in some cases reversibility has failed (Vince-Prue, 1975). More evidence is required before the SD induction of dormancy and the LD releasing of dormancy can be labelled solely as a phytochrome mediated response.

Irrespective of the nature of the photoreceptor, daylength is of major importance to woody species. The use of daylength instead of other environmental factors, such as temperature, to measure and detect seasonal change has two advantages. Firstly, dormancy development involves a period of intense preparation requiring favourable conditions after the receipt of the signal, and so a reliable early warning system is needed to allow the necessary changes to be completed before the onset of winter. The bud needs to be able to enter the winter season already prepared for the adverse conditions. Secondly, the absolute regularity of the timing of daylength cycles from year to year enables shoot growth and development to stop and start with the same regularity. Other environmental factors, e.g. temperature, soil moisture and mineral nutrient levels, and light intensity, can modify this predetermined period and pattern of growth to ensure that the plant is best able to survive or take advantage of the yearly variation in these climatic factors. Although low day temperatures and high night temperatures can induce dormancy (Perry, 1971), temperature as a signal for dormancy initiation is less accurate and predictable than daylength. Nevertheless, photoperiod may not always control the induction of dormancy, even in those species shown to be responsive under experimental conditions. For example, photoperiod response may occur only within a certain temperature range (Moshkov, 1935; Downs and Borthwick, 1956a; Paton and Willing, 1968; Downs and Bevington, 1981), or temperature may completely override photoperiod as shown by Olmsted (1951) with *Acer saccharum* and by Perry (1962) for *Acer rubrum*.

In nature, the initiation of dormancy is probably the result of the interaction between photoperiod, temperature, and soil moisture and nutrient levels. Withholding water, or restricting mineral nutrient

supply, especially nitrogen (Horgan and Wareing, 1980; Darrall and Wareing, 1981), will cause the development of dormancy under experimental conditions, and it is expected that such stresses can accelerate the development of dormancy under natural conditions. However, the buds formed in response to such stresses are quiescent at first and removal of the stress results in renewed growth. However, if the stress is not removed, dormancy is accelerated by SD's under natural conditions. This response to water, nutrient, and temperature stress provides a means for some species to withstand the high temperatures and drought occurring in dry climates. Hence, the external factors, which cause the cessation of growth and induction of dormancy, are different in different species depending on the location of their habitat or place of origin. Some species become adapted to a climate in which the unfavourable season is the hot dry summer, whereas others have become adapted to survival of a cold winter, or a combination of a hot dry summer and a cold winter. The adaptation to these habitats will be reflected in the degree of interaction between the environmental factors which control the respective patterns of growth.

For experimental purposes, the most convenient method of initiating dormancy has been the manipulation of daylength whilst other environmental factors are kept constant.

1.3 ENVIRONMENTAL FACTORS RESPONSIBLE FOR THE RELEASE OF DORMANCY

The state of dormancy, imposed in buds during the autumn, diminishes during the course of winter and this has been shown to be a function of temperature or an interaction between temperature and daylength (see references given in Nooden and Weber, 1978). Although SD treatment causes the cessation of extension growth and the onset of dormancy, placing dormant plants in LD's or continuous light will not result in the resumption of growth, e.g. Wareing (1954) found that no growth had occurred in dormant *Acer pseudoplatanus* and *Robinia pseudoacacia* seedlings after 10 weeks of continuous illumination. The majority of woody species require an exposure to a period of winter chilling to overcome the dormancy of their buds. Temperatures near $5^{o}C$ are most effective in overcoming dormancy, and temperatures above or below the optimum result in a longer chilling period (Campbell and

Sugano, 1975). Temperatures near 0°C and above 15°C are not effective in meeting the chilling requirement, and high temperatures (e.g. above 10°C) can negate the effect of previous chilling, so that the tree sinks back into the original state of innate dormancy (Erez, Couvillon and Hendershott, 1979). Chilling periods of 260-1000 hours are normally required to overcome dormancy (Samish, 1954). The length of the chilling period varies between species and even between varieties of the same species as seen in commercial fruit trees; varieties of apple may require 1000-1400 hours below 7°C (Salisbury and Ross, 1978). In regions having a cold winter, the chilling requirement is met by spring, but in warmer regions or short winters, this is not so and irregular or delayed bud burst occurs. This has been a factor in the establishment of commercial orchards in warm regions with mild winters, such as the tropics, Australia and South Africa. Although chilling is necessary to remove dormancy of buds in many species, warm temperatures are necessary for the growth of the buds following their release. The buds remain in a state of quiescence until temperatures rise, and the actual time of bud burst in spring is marked by the presence of rising temperatures.

The action of chilling in breaking dormancy is not understood, although the assumption is, that it is a cumulative effect and that the required hours of chilling need not be consecutive (Erez and Lavee, 1971; Vegis, 1973). An understanding of chilling is difficult because the interaction of high and low temperatures, the restricted ranges of temperatures above and below which growth cannot be renewed, and the variation with time are most complicated and confusing (Vegis, 1964).

The effect of photoperiod on bud break has been studied in several species. There are species, e.g. *Fagus sylvatica, Larix decidua, Weigela florida* and *Rhododendron* sp., which can always be induced to grow by long photoperiods without any chilling (Wareing, 1953, 1954, 1969; Doorenbos, 1953; Downs and Borthwick, 1956a, b). In these species having no low temperature requirement for dormancy release, the resumption of growth is always dependent on the exposure to LD's. In species which require a chilling period, LD's will break the dormancy of buds only if the buds are in a state of correlative dormancy or quiescence (Wareing, 1956). In species, e.g. *Quercus robur*, where flushes of growth occur during a single growing season, LD's will promote growth between successive flushes but will not overcome the SD-induced dormancy without prior chilling (Wareing, 1956). In some

species, that require chilling prior to bud burst, LD's can substitute partly for the effect of low temperature. For example, in *Betula pubescens*, two to three weeks of continuous illumination will break dormancy (Wareing, 1954). Similarly, dormant buds of *Liquidamber styraciflua* (Downs and Borthwick, 1956b) and *Populus* x *robusta* (Van der Veen, 1951) can be made to resume growth if exposed to continuous light for several weeks and months, respectively. However, when dormancy is fully broken by low temperatures in species having a chilling requirement, the resumption of bud growth is usually independent of daylength and can even occur in continuous darkness (Wareing, 1956).

There appears to be considerable variation between species in their responsiveness to daylength during bud break (Vince-Prue, 1975). It is not clear just how important photoperiod is in the control of bud break in nature but Vince-Prue speculates that photoperiod may be of special importance for the breaking of dormancy in woody species in regions of warm winters. The actual time of bud burst in these and other natural situations would need to be determined by an interaction between photoperiod effects and temperature. Unfortunately the interaction of photoperiod effects and chilling requirements has not been investigated in detail for many species.

In species having photoperiod sensitive buds, enough light is able to penetrate through the bud scales to bring about a response (Wareing, 1953; Pukacki, Giertych and Chalupka, 1980). The apical meristem itself is insensitive to daylength, and Wareing (1969) has suggested that the well developed leaf primordia are the photoperiod perceptors. Furthermore, Wareing (1969) suggests that the difference between species, such as *Fagus sylvatica*, which are dependent entirely on daylength for bud break, and other species relates primarily to a difference in the age at which the leaves become sensitive to photoperiod. Although studies are scarce and incomplete, it appears that the LD breaking of dormancy, like the SD induction of dormancy, is a phytochrome response (see Wareing, 1956).

In addition to the natural removal of bud dormancy by chilling temperatures and LD's, dormancy can also be overcome by applying a wide range of chemical substances and treatments, including ethylene chlorohydrin, thiourea, DNP, gibberellins, cytokinins, and immersion in a water bath at 40°C (see references in Wareing and Saunders, 1971).

In many of these cases, it is unclear as to what degree the chilling requirement was already satisfied before the chemical treatment was begun. Therefore, it is not clear whether these substances accelerate growth or whether innate dormancy is broken by the treatment.

1.4 HORMONAL CONTROL OF DORMANCY

At the end of the nineteenth century it was suggested by Sachs that growth and differentiation within the plant involved chemical messengers or more specifically "organ forming substances" (see Letham, Higgins, Goodwin and Jacobsen, 1978; Heslop-Harrison, 1980). The study of phototropism in coleoptiles of cereals led to the discovery of auxin, and the work that followed confirmed the concept that plant growth and development were under hormonal control (for a historical account see Went and Thimann, 1937). The environmental factors controlling plant growth were thought to operate through a hormonal mechanism. The observation (Wareing, 1954), that leaves exposed to SD's have an inhibitory influence on the growth of buds of some woody species, suggested that some transmissible, chemical substance or growth inhibitor may be involved in the phenomenon of bud dormancy. In order to find such a substance and prove its role as a regulator of bud dormancy, researchers followed two complementary experimental approaches. The first sought to establish a correlation between levels of endogenous growth substances present in leaves, stems, and buds with the state of growth or dormancy within the plant. Corroborative evidence, of any positive or negative correlation found, was provided by the second approach, which was to apply exogenous growth regulators to various plant tissues at various stages of dormancy to induce, maintain or delay dormancy.

Early reports (reviewed by Wareing and Saunders, 1971) of experimentation using either or both of these approaches, suggested that certain phases of dormancy involved some degree of hormonal control. Wareing's inhibitor hypothesis in which abscisic acid was implicated either solely or in conjunction with growth promoters such as gibberellins, was regarded as the hormonal mechanism involved in the control of dormancy. However, since then a large body of conflicting evidence has made it impossible to formulate any general hypothesis on

the regulation of bud dormancy by any or all of the groups of hormones, that are known to occur in plants.

Presently, five groups of hormones, auxins, gibberellins, cytokinins, growth inhibitors (specifically abscisic acid) and the gas, ethylene, are recognised as occurring in plants and functioning as controllers of growth and development (Letham, Goodwin and Higgins, 1978). Although dormancy is a developmental phase in its own right, it was considered to be an inhibited state of growth and hence much research has been done on growth inhibitory compounds, especially abscisic acid. The evidence from such research (Wareing and Saunders, 1971) has suggested growth inhibitors are implicated in the regulation of dormancy. However, other hormones have also been studied and suggestive roles within bud dormancy assigned to them.

1.4.1 Growth Inhibitors

Growth inhibitory substances, generally, refer to those substances, in plant extracts, which inhibit the extension growth of cereal coleoptiles. Initially, these substances were detected by Went's *Avena* curvature test (Went and Thimann, 1937), which was being used to detect auxin or auxin-like growth promoters. However, the more convenient *Avena* mesocotyl and *Avena* coleoptile section assays (Nitsch and Nitsch, 1956) and the *Triticum* coleoptile section assay (Bentley and Housley, 1954) are more commonly used to detect such compounds in plant extracts.

The occurrence of growth-inhibiting substances in buds of woody species was first reported by Hemberg (1949b). Hemberg found that there was a greater amount of growth inhibiting substances in resting than in non-resting terminal buds of *Fraxinus*. Furthermore, breaking the dormancy of buds by chemical treatments resulted in lower amounts of growth inhibitory substances in the buds. From these observations, Hemberg postulated that growth inhibiting substances, as detected by the *Avena* curvature test, were of significance in the control of the rest of terminal buds of *Fraxinus*. The presence of a growth inhibitor in dormant peach flower buds (Hendershott and Bailey, 1955) and maple buds (Steward and Caplin, 1952), and the marked decrease in the level of this inhibitor or inhibitors at the end of the dormant season, added support to the findings of Hemberg.

The growth inhibiting substances extracted by Hemberg were ether and water soluble, and acidic in nature. They were termed inhibitory because of their ability to inhibit the bending of coleoptiles in the *Avena* curvature test (Went and Thimann, 1937). However, fractionation of the plant extracts was not attempted and so promoters as well as inhibitors were present in these extracts and those made by Steward and Caplin (1952) and Hendershott and Bailey (1955). The interpretation of the results were, therefore, in question. It was possible that the disappearance and reappearance of growth promoters occurred rather than a change in the levels of growth inhibitory substances.

Luckwill (1952) and Bennet-Clark, Tambiah and Kefford (1952) reported the use of paper chromatography to fractionate plant extracts into growth promoters (auxin-like compounds) and growth inhibitors. The major acidic, ether-soluble inhibitors from plant tissues migrated to zones with R_F's greater than 0.5, whereas the growth promoters remained within zones located at R_F's less than 0.5, when developed in isopropyl alcohol : water : ammonia :: 10:1:1 solvent. The zone of growth inhibition, as detected by *Avena* coleoptile test, was termed Inhibitor β by Bennet-Clark and Kefford (1953).

Blommaert (1955) used the paper chromatography technique and found that acidic growth inhibitors play a role in the regulation of the rest period in peach buds. Similarly, Phillips and Wareing (1958) made use of paper chromatography techniques, and studied growth substances present in buds and leaves of sycamore (*Acer pseudoplatanus*) throughout the annual cycle of growth. An inhibitor, comparable to inhibitor β and running at R_F 0.7, showed quantitative changes in extracts harvested during the course of the year. Greatest inhibitory activity was detected in extracts of the apical region, that were harvested in autumn or early winter, and the least activity at the time of active growth in early summer. The inhibitor content of apices showed a gradual decline during the winter and increased during late summer and early winter, whereas the inhibitor content of mature leaves increased during late summer - autumn period. The extracts were assayed on wheat coleoptiles, lettuce seeds, cress roots, and leaf discs of sycamore, and therefore, demonstrated that the growth of sycamore tissue itself was influenced by the inhibitor or inhibitors within the Inhibitor β fraction. It was suggested, on the basis of the correlation between growth or dormancy

state and inhibitor level, that the inhibitor was responsible for the regulation of dormancy. Furthermore, it was suggested that the inhibitor was produced in the leaves under the influence of SD's and transported to the apex where it accumulated during autumn and decreased during the course of winter as a result of chilling. Earlier, Wareing (1954) had shown that seedlings of *Acer pseudoacacia* and *Betula pubescens* show a marked photoperiod response in relation to dormancy with SD's bringing about the cessation of extension growth and the formation of dormant buds. The results, then, were consistent with the view that photoperiod regulated the level of inhibitor β present in the buds and therefore controlled dormancy.

However, in the natural stands of sycamore used by Phillips and Wareing (1958), the effect of photoperiod on the growth inhibitor content of extracts was possibly complicated by the effects of other seasonal variables. To confirm the effect of photoperiod on growth inhibitor levels, Phillips and Wareing (1959) used sycamore seedlings and controlled environment conditions, and found that higher levels of inhibitor β were present in both apices and mature leaves of plants transferred to SD conditions than those maintained under LD's. Furthermore, this increase preceded any marked effect of daylength treatment on extension growth and was detected in mature leaves after only two cycles of SD treatment. The increase in apices occurred later and was detectable after five cycles of SD treatment. The evidence from the studies on sycamore (Phillips and Wareing, 1958, 1959) were consistent with a view that inhibitors produced in the leaves, under the influence of SD's and transported to the shoot apices, caused and regulated the development of dormancy.

More conclusive evidence in support of the inhibitor hypothesis came from studies on *Betula pubescens* (Eagles and Wareing, 1963, 1964). Actively growing birch seedlings were induced to form resting buds under non-inducive conditions by immersion of a young expanded leaf in an aqueous solution of the partially purified inhibitor, which had been extracted from leaves of dormant birch seedlings. The successful induction of dormancy in birch seedlings provided crucial evidence for the theory that inhibitors play a regulatory role in bud dormancy of some woody species. The term "dormin" was suggested (Eagles and Wareing, 1963, 1964) for substances which function as endogenous dormancy inducers and regulators as distinct from general growth inhibitors.

Further work, on the isolation and determination of the chemical nature of "dormin", was carried out (Robinson, Wareing and Thomas, 1963; Robinson and Wareing, 1964) and it was found that dormin was identical with abscisin II, an abscission accelerating substance, isolated from cotton plants (Cornforth, Milborrow, Ryback and Wareing, 1965). The inhibitor was later renamed abscisic acid (ABA) (Addicott, Carns, Cornforth, Lyon, Milborrow, Ohkuma, Ryback, Smith, Thiessen and Wareing, 1968).

The availability of synthetic ABA (Cornforth, Milborrow and Ryback, 1965) allowed the crucial experiment of dormancy induction by exogenous application of inhibitor to be repeated, specifically using ABA. It was found that synthetic ABA induced bud dormancy in actively growing seedlings of *Betula pubescens*, *Acer pseudoplatanus* and *Ribes nigrum* when applied in aqueous solution to the leaves. Such treatment brought about the cessation of extension growth, and the formation of resting buds with apparently normal bud scales (El Antably, Wareing and Hillman, 1967). At this stage, the evidence in favour of the inhibitor β or ABA theory of dormancy seemed beyond refute and unassailable. A large number of species and tissues had been studied and the role of inhibitor β seemed beyond question from the accumulated evidence.

Blommaert (1955, 1959) found that dormant peach buds had a high growth inhibitor content during the winter and that the content fell as winter progressed. The inhibitor level dropped at a more rapid rate in trees subjected to a cold dormant period than that in trees exposed to a warm dormant period. The inhibitor was shown to have an R_F similar to inhibitor β. Increased activity of the β fraction during the development of dormancy was demonstrated in sycamore maple by Dorffling (1963a,b) thus confirming the work of Phillips and Wareing (1958). Inhibitor β levels were measured in buds, leaves, and cambial tissue at different stages of development and at various times during the seasons. Inhibitor β was detected in all three tissues with terminal buds showing an accumulation of inhibitors during the summer-autumn induction period and a fall in inhibitor content during the time of bud burst. In dormant shoots, the inhibitor content decreased from tip to base, whereas in growing shoots the inhibitor content decreased from base to the top. Therefore, the shoot system possesses a gradient of inhibitor activity that changed with dormancy status. Eliasson (1969) showed that there is a marked increase in the inhibitor β content of leaves of aspen

(*Populus tremula* L.) harvested when growth had ceased in October compared with those harvested the previous autumn. He concluded that inhibitor β in aspen was of importance for the development of the dormant state. A decrease in levels of inhibitor β in buds at the time of bud burst, in the spring, was demonstrated in terminal buds of *Fraxinus* (Hemberg, 1958a), sycamore maple (Guttenberg and Leike, 1958), *Pinus palustris* saplings (Allen, 1960), and red pine (Giertych and Forward (1966). Similarly, Kawase (1966) found that the level of inhibitor β was at a maximum during the winter season, but gradually declined thereafter towards spring, and that, breaking of bud dormancy in four woody species, *Diospyros virginiana* L., *Malus sylvestris*, *Prunus persica* and *Ulmus americana* L., was accompanied by the disappearance of inhibitor β from the buds. Evidence on the seasonal changes in inhibitor β activity of leaves and buds, therefore, indicated a causal relationship between inhibitor β and dormancy.

The increase in inhibitor β content of buds and leaves of seedlings, transferred from LD's to dormancy-inducing SD's, reported by Phillips and Wareing (1958), has been demonstrated by other workers. A study of *Betula pubescens* and *Betula lutea* by Kawase (1961), revealed that the concentration of inhibitor β, obtained by methonal extraction or diffusion from excised apices, increases in apices as the number of SD's increases. On returning plants to LD's the amount of inhibitor was found to gradually decrease until the resumption of growth occurred. The increase in inhibitor content of apices occurred after elongation had ceased but there was an early increase in the stems. Dormancy-inducing SD's increased the inhibitor activity in buds of staghorn sumac (*Rhus typhina* L.) (Nitsch, 1957a) and flowering dogwood (*Cornus florida* L.) (Waxman, 1957). Members of Wareing's group at Aberystwyth have also reported increases in inhibitor β in apices and leaves due to SD treatments. Robinson, Bowen and Thomas (cited in Wareing and Saunders, 1971) found an increase in tissues of *Acer pseudoplatanus*, *Salix viminalis* and *Betula pubescens*, respectively. Hoad (1967) also reported that SD's led to a marked increase in the inhibitor β content of phloem exudate of *Salix viminalis* cuttings.

Since the discovery of ABA and the advent of positive methods of identification and measurement of ABA, several studies, on endogenous levels of ABA in tissues of field grown woody species, have indicated that ABA increases during dormancy development, and decreases during the

time of bud burst. In a study of the ABA content of buds and nodes of di- and tetraploid varieties of grapes, it was found by During and Kismali (1975) and During and Bachmann (1975) that ABA levels increased during dormancy development, with a maximum occurring at the time of minimum bud burst in October, and then decreased in November-December, when all varieties showed increasing bud burst. The use of budded cuttings, placed under environmental conditions favourable for growth, enabled these workers to precisely determine the periods of pre-dormancy, endogenous or innate dormancy and imposed dormancy. From this work, it was established for *Vitis vinifera*, that decreasing and increasing capacity for bud burst, in several varieties, was correlated with changes in the ABA level of buds; there being an inverse relationship between capacity for bud burst and ABA content. The content of ABA in blackcurrant (*Ribes nigrum*) and beech (*Fagus sylvatica*) buds also shows a seasonal fluctuation (Wright, 1975). The highest level of ABA was observed in the autumn at about the time of onset of dormancy, and thereafter the ABA content of the buds declined throughout the winter to reach its lowest level just before bud burst. Bowen and Derickson (1978) reported that the chilling requirement of peach flower buds from seven clones were well correlated with ABA levels at the time of deep rest in December. Those clones possessing a greater chilling requirement had more ABA in the buds than those clones with lower chilling requirements at this time. Recently, Orchard, Collin and Hardwick (1980) have presented data to substantiate a role for ABA as a determinant of the dormant phase of the flush cycle in cocoa (*Theobroma cacoa*). There was a correlation between the activity of the terminal apex and the content of ABA in the leaves. They suggested that leaves of the current and previous flush act as a source of ABA, which maintains the apical bud in a state of dormancy. The level of ABA in the leaves was found to fall prior to bud break. Numerous other studies have also shown that the ABA content of buds and other tissues decreases at the time of bud burst in many species including peach (Corgan and Peyton, 1970; Corgan and Martin, 1971), blackcurrant (Kuzina, 1970), coffee (Browning, Hoad and Gaskin, 1970), apricot (Ramsay and Martin, 1970), willow (Michniewicz and Galoch, 1972), grape (During and Alleweldt, 1973), poplar (Bachelard and Wightman, 1974) and *Abies sachalinensis* Masters (Shibakusa, 1977). Recently, Webber, Laver, Zaerr and Lavender (1979) and Dumbroff, Cohen and Webb (1979) studied the seasonal variation of abscisic acid in the dormant shoots of

Douglas fir and in buds and stems of *Acer saccharum*, respectively, and found that there was an increase in ABA levels in these tissues over the winter period followed by a decline in ABA levels at the time of bud burst. The above results from seasonal studies on ABA levels in buds of a wide range of woody species support and strengthen the view that ABA has an important role in the induction and maintenance of dormancy.

Further evidence in support of this view has come from studies on the levels of inhibitor β and/or ABA that are present in the xylem and phloem. The demonstration of the existence of inhibitor β and ABA in these tissues is, of course, consistent with Wareing's (1969) view that ABA is produced in the leaves under SD's and moves to the apices causing dormancy. A marked seasonal variation in the inhibitor content of xylem sap collected from *Salix fragilis* was demonstrated by Davison (1965); inhibitor levels were higher during the dormant winter period than in midsummer. Similarly, Bowen and Hoad (1968) found that the inhibitor β content of phloem and xylem sap increased as willow (*Salix viminalis*), grown under natural conditions in the field, entered dormancy. Earlier, Hoad (1967) had established the presence of ABA in the phloem exudate, which had been collected using aphids. An autumn peak in the ABA concentration, followed by a decrease at the time of visible bud swell in the xylem sap of *Prunus persica* cv. Golden Queen was shown by Davison and Young (1974). Alvim, Hewett and Saunders (1976) followed changes in levels of inhibitor β and ABA in the xylem sap of willow (*Salix viminalis*) throughout two growth cycles and found marked seasonal changes. There was an increase in inhibitor β and ABA concentration in the autumn and a subsequent fall in the spring. Furthermore, another peak in the inhibitor β and ABA concentration of the sap, leaves and apices was found shortly after midsummer, immediately preceding the cessation of shoot extension. These changes in inhibitor β and ABA were well correlated in time with growth rate and dormancy. Harrison and Saunders (1975) noted that a decrease in the ABA level in the xylem sap of birch (*Betula verrucosa*) occurred as bud dormancy was broken naturally. Similarly, the ABA level in the bleeding sap of birch (*Betula pubescens* Ehrh.) was also found to decrease during bud burst in the spring (Dathe, Sembdner, Kefeli and Vlasov, 1978; Vlasov, Kefeli, Artemenko, Umnov, Sembdner and Dathe, 1978). The main acidic inhibitor in the bleeding sap was identified as ABA. This group of workers reported that the level of ABA in wood (mainly xylem) and

bark (mainly phloem) was high during dormancy and low after bud burst. During bud burst the level of ABA in the wood fell, whereas the bark showed a rise in ABA content. The inhibitor content of phloem exudate of willow (*Salix viminalis*) was shown to be influenced by daylength (Hoad, 1967). There was more abscisic acid in aphid honeydew collected from plants transferred to dormancy inducing SD's than those kept under LD's. In the above studies on the inhibitor content of xylem and phloem saps, no data on the rates of movement of the sap were made, and therefore, it is difficult to relate concentrations measured with the actual amount reaching the apex. Nevertheless, the demonstration of the occurrence of ABA in the xylem and phloem sap adds some support to the inhibitor hypothesis.

The availability of synthetic ABA has facilitated many studies on the effects of exogenous applications of ABA on bud dormancy. Besides the report of El Antably et al. (1967), there are no reports in the literature that show exogenous applications of ABA can cause the induction of bud dormancy in woody species. However, the ability of ABA to delay bud burst, when applied exogenously near the time of natural bud burst, has been reported for several species, and this evidence has been taken as being supportive of the inhibitor hypothesis. El Antably et al. (1967) reported that application of ABA to buds, by the immersion of the cut bases of stems, effectively delayed bud burst in *Ribes nigrum* and *Salix viminalis*. Similarly, Little and Eidt (1968) found ABA delayed bud break in cuttings of the diffuse porous species, white ash (*Fraxinus americana*) and red maple (*Acer rubrum*), as well as in the coniferous species, white spruce (*Picea glauca* (Moench) Voss) and balsam fir (*Abies balsamea* L.). Similarly, Haissig and King (1970) found that exogenous applications of ABA effectively inhibited bud break in seedlings of white spruce. Bud outgrowth in coffee (Van der Veen, 1968) and Redblush grapefruit seedlings (Young and Cooper, 1969) was delayed by exogenous ABA applications. Cohen and Kelley (1974) showed that immersion applications of ABA effectively delayed bud break and inhibited shoot growth in dormant plants of *Rosa* and *Syringa*. The incubation of excised buds isolated from dormant maple trees, on aseptic media containing ABA, resulted in the inhibition of bud burst (De Maggio and Freeburg, 1969). Using similar *in vitro* techniques Altman and Goren (1974a,b), who used citrus buds, and Dutcher and Powell (1972) and Singha and Powell (1978), who used apple (*Malus domestica* Borkh cv Northern

Spy) buds, found that ABA inhibited bud break and shoot elongation. The ability of ABA to delay bud burst near the time of natural burst is, then, well documented.

In addition to the regulation of bud growth, ABA has also been shown to influence or be implicated in the growth activity of the stem and roots of some woody species. Working with young larch trees, Wodzicki (1965) showed that there was a correlation between the seasonal peak of a water soluble inhibitor and latewood formation at the end of the growing season. From a study on forest grown radiata pine (*Pinus radiata* D. Don) trees, Jenkins and Shepherd (1974) concluded that cambial activity at times of moisture stress was influenced by ABA. The ABA content of stem tissue was measured throughout one annual cycle. Fluctuations in the content of ABA occurred during the summer-autumn period with peaks coinciding with times of moisture stress. During the late autumn and winter, the levels of ABA present in the stem were low and cambial growth showed some correlation with the levels of ABA present. This is in accordance with the theory of growth quiescence. *Pinus radiata* lacks true dormancy as known in many other trees of the temperate zone, and its growth capacity is limited by the degree of favourability of the environmental conditions (Barnett, 1971; Skene, 1969). More recently, Wodzicki and Wodzicki (1980) reported a seasonal accumulation of abscisic acid in the stem region of *Pinus silvestris* and these authors discuss the contribution of ABA to the hypothesis of a late wood control system in conifers. The seasonal growth activity of roots of woody species has also been correlated with ABA levels. Cohen, Dumbroff and Webb (1978) found that the seasonal pattern of abscisic acid in roots of *Acer saccharum* was suggestive of ABA having a key regulatory role in the growth cycle of roots. Low levels of ABA were associated with periods of maximum root growth, prior to bud break in the spring and during the fall. During the period of most active shoot growth, a 25 fold increase in ABA was detected in roots coinciding with a marked decrease in root elongation rates. A recent report (Philipson and Coutts, 1979) established the ability of ABA to induce dormancy in roots of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) cuttings growing in solution culture. Dormancy was characterised by the superficial browning and the development of a layer of lignified and suberised cells around the root apex, as seen in naturally induced root dormancy.

The examination of the literature, as reported above, indicates that the inhibitor hypothesis has a basis in a wide range of tree species. The evidence in these reports supports the inhibitor hypothesis that ABA is causally involved in the regulation of the cycle of shoot growth and bud dormancy in woody species. However, there are also several reports that indicate inhibitor β and ABA may not have a causal role in bud dormancy. The expected rise in the inhibitor β levels in response to photoperiodic induction of dormancy did not occur in *Betula pubescens* and *Robina pseudoacacia* (Phillips and Wareing, 1958, 1959). However, increased inhibitor levels under SD's were subsequently demonstrated in *Betula* by Kawase (1961), and Eagles and Wareing (1963). Eliasson (1969) showed that the inhibitor β content of aspen (*Populus tremula*) shoot apices did not change markedly after 13 days of SD treatment, although shoot growth had just ceased at this time. A lack of correlation between inhibitor β content of buds and dormancy status has also been reported to occur at the time of bud break. No change in inhibitor levels or a change only after buds had visibly emerged from dormancy, has been reported for lilac (*Syringa vulgaris*) buds (Guttenberg and Leike, 1958), English oak (*Quercus pendunculata*) buds (Allary, 1960, 1961) and apple buds (Pieniazek, 1964b; Pieniazek and Rudnicki, 1971). Taylor and Dumbroff (1975) reported that the inhibitor β content of sugar maple (*Acer saccharum*) buds remained relatively constant during the entire dormant period and no decrease was found at the time of bud break. In peach floral cups, Corgan and Martin (1971) showed that the level of an inhibitor, which was thought to be ABA, fluctuated during the winter months with a peak occurring just prior to the termination of dormancy. Hence, for certain species, it has been found that there were no changes in inhibitor β activity that could be correlated with changes in the dormancy status of buds.

Investigations in which the ABA content of leaves and buds were determined by relatively specific methods, have also shown the lack of an observed trend in ABA activity, that could be correlated with changes in bud dormancy, under natural field conditions. Emmerson and Powell (1978), who worked with several cultivars of three species of *Vitis*, found that ABA decreased during winter in all four cultivars investigated. However, there was a much higher ABA content in non dormant lateral buds (those committed to grow in the year of formation) than adjacent axillary buds (those requiring a chilling period) of

Vitis labruscana. Furthermore, they found that ABA levels appeared to increase in buds and bud primordia as bud burst progressed. More recently, Alvim, Thomas and Saunders (1978) studied the variation in the abscisic acid levels of field grown willow apices throughout the summer months and found that a peak in ABA occurred some two to three weeks before growth cessation. Natural photoperiod was not changing rapidly at this time. Similarly, Mielke and Dennis (1978), working with sour cherry buds, showed that an autumn peak in ABA content was not correlated with the onset of dormancy but with the onset of leaf abscission. Furthermore, artificial defoliation prevented the increase of ABA in the buds, but did not affect the intensity of rest possessed by the buds. Therefore, these authors concluded that the higher ABA content of buds during autumn was not associated with the imposition of dormancy. In a recent study, Dumbroff, Cohen and Webb (1979) measured the ABA content of sugar maple (*Acer saccharum*) buds at approximately monthly intervals during the year. No accumulation of ABA occurred during the period of dormancy development in late summer and early autumn. The level of ABA was, in fact, lower than in the preceding summer months. However, as has been demonstrated for other species, there was a peak in ABA concentration coinciding with the period of deepest dormancy and a subsequent decline in ABA levels as winter progressed, with the lowest ABA levels occurring shortly before bud swell and bud burst. Therefore, these workers concluded that ABA may have a significant function in the overwintering process or dormancy maintenance but not in dormancy induction.

Evidence from other studies, however, indicates that changes in ABA Levels are not correlated with the chilling requirement of buds or the acceleration or delay of bud burst by artificial means. For example, Mielke and Dennis (1978) in a study of winter bud dormancy of sour cherry (*Prunus cerasus*) found that once the level of ABA had risen naturally in the autumn, temperature had no effect on its disappearance. The ABA content of the flower primordia was found to decrease irrespective of whether plants were left outdoors or placed in a greenhouse, at either $4^{o}C$ (optimum chilling temperature) or $22^{o}C$. Since ABA decreased in chilled and unchilled buds, the satisfaction of a chilling requirement does not appear to involve a decrease in the content of free abscisic acid. In another study of bud break and ABA levels, Iwasaki and Weaver (1977) used cuttings of 'Zinfendel' grape

(*Vitis vinifera*) and found that calcium cyanamide (CaCN) treatment and storage at $0^{o}C$ accelerated bud burst compared with untreated buds similarly stored. However, changes in ABA content did not correlate with bud response in that the CaCN treated buds had a higher ABA concentration than untreated buds. A delay in bud break of Redglobe peach (*Prunus persica*) trees was obtained by nitrogen (N) applications to the soil during the previous spring and autumn (Reeder and Bowen, 1978). The delay in bud break was not correlated with ABA levels in that the levels of ABA in buds of trees given the highest nitrogen treatment were lower than those of buds from trees receiving the lowest nitrogen treatment. Therefore, there exists a number of reports in which the ABA content of buds is not correlated with their dormancy status under field conditions.

The presence of a bound or conjugated form of ABA, namely abscisyl-β-D-glucopyranose, in a variety of plant tissues (Milborrow, 1978a) including dormant grape buds (During and Alleweldt, 1973), has led to several studies in which changes in abscisic acid as well as bound ABA were determined throughout the dormancy cycle. Bound ABA is often referred to as alkali-labile or alkali-hydrolysable conjugates as treatment with mild alkali releases ABA as the free acid. It is thought that the conjugated or bound form of ABA is a storage or inactive form, and that by interconversion to the free acid, it may play a role in regulating the level of free or active ABA present in plant tissue. With this possibility in mind, several studies have reported seasonal fluctuations in both free and bound ABA. Wright (1975) determined the levels of free and bound ABA in blackcurrant (*Ribes nigrum*) and beech (*Fagus sylvatica*) buds by a bioassay method. The buds of blackcurrant showed a rapid decline in free ABA with a concomitant, though smaller, increase in conjugated ABA during early autumn. These levels were maintained over the winter period until early spring when the amount of conjugated ABA increased appreciably. The ABA content of beech buds showed similar changes to that of blackcurrant buds but differed, in that the decrease in free ABA following the autumn peak was not accompanied by a rise in the levels of conjugated ABA. The ratio of free ABA to bound ABA showed a distinctive annual cycle, with the highest free ABA to bound ABA ratio occurring in the autumn followed by a progressive decline until the time of bud burst when the ratio of bound ABA to free ABA was the highest. Harrison and

Saunders (1975) reported similar findings for *Betula verrucosa* buds. There was no clear progressive decline in ABA during the winter, but the ratio of free ABA to bound ABA decreased as the buds became less dormant. The sharp increase in conjugated ABA could not be accounted for by a corresponding decrease in free ABA. It was suggested that the emergence from dormancy was associated with an increased capacity to esterify or conjugate free ABA. Whilst these two reports strengthen the view that ABA plays an important role in the regulation of bud dormancy, other reports indicate discrepancies between changes in the levels of free and bound ABA and the state of dormancy in buds. For example, in sour cherry flower buds, the level of bound ABA tends to parallel that of free ABA (Mielke and Dennis, 1978). Maximum levels in free and bound ABA occurred when 90 to 95 per cent of the leaves had abscised and not at the time of onset of dormancy. In addition, the level of free ABA returned to its original level several weeks prior to the termination of dormancy and was not correlated with the period of deepest dormancy. Bound ABA decreased slowly during the winter and at the time of bud burst, no increase in bound ABA levels occurred. These observations argued against a role of free and bound ABA in the control of bud dormancy of sour cherry. More recently, Phillips and Hoffman (1979) measured the changes in levels of free and bound ABA in terminal buds of *Acer pseudoplatanus*, during natural emergence from winter dormancy, using gas chromatographic techniques. No changes in free ABA or bound ABA occurred until after the buds had been released from true dormancy by winter chilling. There was, however, a large transient increase in a metabolite of ABA, phaesic acid (PA), but this was not accompanied by any marked decrease in either free or bound ABA. It was concluded that glycosylation of ABA does not play a major part in the mechanism of release from dormancy, and that the disappearance of dormancy in the buds cannot be ascribed to a reduction in overall levels of ABA. Weiler (1980) used a highly specific radio-immunoassay technique to measure free and conjugated ABA in buds and leaves of *Betula papyrifera* and *Acer pseudoplatanus*. In both species, Weiler was unable to find any correlation between the seasonal variation in the ratio of free to conjugated ABA and the dormancy status of the buds.

No significant changes in either free ABA or bound ABA occurred during the winter in buds of almond (*Prunus amygdalus* cv. Pariche) trees (Leshem, Philosoph and Wurzburger, 1974). However, the level of the

trans, trans isomer of ABA (trans ABA) decreased and its corresponding conjugate increased with the emergence from dormancy. On the basis of this observation, they postulated that conjugation of the free trans trans stereoisomer of ABA is a contributing factor in bud break. Similarly, Jones, Coggins and Embleton (1976) have suggested a role for trans ABA in the control of bud dormancy of alternate bearing 'Valencia' orange (*Citrus sinensis* L. Osbie) trees. They found five to ten fold more trans ABA than cis trans ABA in the dormant buds and a dramatic drop in both stereoisomers as spring growth approached. No bound ABA was detected. Unlike Leshem et al. (1974), Jones et al. (1974) used gas chromatography-mass spectroscopy to confirm the identity of the trans trans isomer. The reported presence of trans ABA in citrus buds, and possibly almond buds, is in contrast to most reports on studies on endogenous ABA in plant tissues where the principal or only isomer found has been the cis trans isomer (Addicott and Lyon, 1969; Milborrow, 1974, 1978a; Zeevaart, 1979. Bearder, 1980). In view of this and the fact that trans ABA is largely inactive in several biological systems (Milborrow, 1966, 1974, 1978a), any suggestion of a role for trans ABA in dormancy control must be viewed with some scepticism. Nevertheless, the studies of Leshem et al. (1974) and Jones et al. (1976), like the studies of Mielke and Dennis (1978), Harrison and Saunders (1975), Phillips (1979) and Weiler (1980), do show that the levels of free and bound ABA in buds are not correlated with the dormancy status of the buds.

It appears from several reports in the literature, that although seasonal fluctuations in levels of ABA occur in buds and leaves, these changes in ABA levels are not always correlated with the development and/or maintenance of bud dormancy under natural field conditions. It is, therefore, not possible to make a generalisation as to the mechanism or role of ABA in the phenomenon of bud dormancy. Controlled environment studies involving the photoperiodic induction of dormancy also indicate that ABA may not play a regulatory role in bud dormancy. The exposure of seedlings to short day dormancy-inducing conditions caused the cessation of extension growth and the formation of dormant buds, but no increase in endogenous ABA levels was found. Lenton, Perry and Saunders (1972) transferred three different species of photoperiodically-sensitive tree seedlings; birch (*Betula pubescens*), red maple (*Acer rubrum*) and sycamore (*Acer pseudoplatanus*), from LD's

to SD's and found no increase in the amount of ABA in leaf extracts, and a small reduction in extracts of apical tissue. They concluded that the photo-induction of bud dormancy was not mediated by changes in ABA levels alone. Furthermore, using radioactively labelled ABA, Loveys, Leopold and Kriedemann (1974) found that there was no difference in the metabolism of ABA in leaves of birch (*Betula lutea*) under LD's or SD's. The levels of radioactive ABA and two of its metabolites, including one in the alkali-hydrolysable fraction, were similar in both treatments. The endogenous ABA content of leaves was found to be lower in leaves of SD treated plants than leaves of LD treated plants. Similarly, dormancy-inducing photoperiods were shown to have no effect on the endogenous ABA levels in apple (*Malus domestica* Borkh cv Northern Spy), tea crabapple (*Malus trupehensis* (Pamp.) Rehd.) and canoe birch (*Betula papyrifera* Marsh.) buds (Powell, 1976). The recently published work of Alvim, Thomas and Saunders (1978) indicates that ABA is not involved in photoperiodically mediated dormancy of willow (*Salix viminalis*). Artificially extended photoperiods, applied to field grown willow plants during the late summer months, prevented the onset of dormancy but the concentration of ABA in the xylem sap, mature leaves, and apices was higher than in the corresponding tissues of plants which had entered dormancy under natural daylengths. Earlier, Saunders, Harrison and Alvim (1974) had shown that rooted cuttings of *Salix viminalis*, maintained under dormancy-inducing photoperiods in growth chambers, ceased shoot elongation and aborted the growing apices, although no increase in endogenous ABA levels occurred within the apices. More recently, Alvim, Saunders and Barros (1979), using rooted cuttings of *Salix viminalis*, found no change in the inhibitor β and ABA content of extracts of root, leaf, apical tissue, and xylem and phloem sap upon transfer of cuttings from LD's to SD's. Similarly, in a study of photoperiod effects on ABA levels in *Acer pseudoplatanus*, Phillips, Miners and Roddick (1980) found that growing plants under LD's or SD's did not influence the levels of free and bound ABA in leaves, although the levels of bound ABA were lower at the end of the dark period, of both LD and SD treatments, than 8 hours later during the light period. In contrast to these studies, where the photoperiodic induction of dormancy had no effect on endogenous ABA levels, Perry and Hellmers (1973) in a detailed study on *Acer rubrum*, found that ABA did accumulate in the levels of two races of maple, in response to treatment with short

photoperiods and cold nights. The two races of maple, Northern (from Massachusetts) and Southern (from Florida), had similar ABA levels in their leaves under LD's and SD's, but only plants of the Northern race ceased growth and developed dormancy. The endogenous ABA content of dormant buds of the two races growing in their natural habitat (i.e. Massachusetts and Florida) were also similar. From the controlled environment studies just mentioned, it appears that dormancy-inducing photoperiods do not always cause an increase in the ABA content of leaves and buds or if endogenous ABA levels are increased, they do not necessarily result in the formation of dormant buds. The role of ABA as a dormancy-inducing hormone must, therefore, be questioned.

From the survey of the literature presented above, evidence conflicting with the inhibitor hypothesis has come from studies on the endogenous inhibitor β and ABA contents of buds and leaves. The lack of agreement between studies on inhibitor β content of buds, in which a correlation between levels of inhibitor β and dormancy status was established, and those in which no such correlation was found, may be explained on the basis of the techniques used to measure inhibitor β. The inhibitor β content of plants can only be determined by bioassay methods, which all have specificity and dose-response limitations. A major difficulty lies in the unknown nature of the extracts tested. In crude extracts, e.g. the acidic ether fraction or in partially purified extracts such as the β fraction, the presence of promoters will offset the activity of the inhibitors and produce an underestimate of the inhibitor activity. Conversely, the presence of several inhibitors, some of which may not be active in dormancy but active in the bioassy either singularly or synergistically, will result in an overestimate of the inhibitory activity in the extract. The Inhibitor β complex obtained by paper chromatography of the acid ether-soluble fractions of plant extracts (Bennet-Clark and Kefford, 1953) does not refer to a specific compound but a zone of growth inhibition found at about R_F 0.5 - 0.8. This zone of growth inhibition is the result of all substances, including any promoters, that are active in the bioassay. Therefore, the failure to separate the promoters from inhibitors and the presence of other interfering inhibitors, may account for the reports in which no correlation between levels of inhibitor β and dormancy of buds was found. On the other hand, in those reports where the decrease in inhibitor activity of buds was

found at the time of dormancy release or an increase at the time of onset of dormancy, in accordance with the inhibitor hypothesis may only reflect increased or decreased promoter activity co-chromatographing with the inhibitors. A partial solution to the presence of interfering substances is the use of more than one type of bioassay and/or the use of serial dilutions. However, this time consuming approach is rarely reported to have been used. Rather, it was accepted (Wareing and Saunders, 1971) that some discrepancies between inhibitor β studies occurred because of the use of bioassays, and that there exists a correlation between the seasonal inhibitor β content of buds and their dormancy status. Such was the attraction of the inhibitor hypothesis.

However, a serious anomaly to the inhibitor hypothesis was raised by the discovery that ABA in buds and leaves did not increase during the photoinduction of bud dormancy (Lenton et al., 1972). This anomaly raised the possibility that the seasonal pattern of inhibitor β, established by earlier studies on which the inhibitor β hypothesis was based, may not reflect, solely, the changes in endogenous ABA. Although ABA is the major component and accounts for most of the inhibitory activity of the inhibitor β fraction of extracts (Robinson and Wareing, 1964; Milborrow, 1967), other inhibitory compounds including phenolics, also occur within the β fraction (Varga, 1957; Varga and Ferenczy, 1957b; Lane and Bailey, 1964; Saunders, 1978b). Clearly, in the early studies where inhibitor β levels were determined by bioassays, ABA would have contributed to the inhibitory activity. However, in no study was it proven that the variation in inhibitory activity was due entirely to a single compound. The possibility remains, that variation in inhibitory activity is due to a change in the amounts of other inhibitors or promoters which overcome the effects of ABA in the bioassay used. Therefore, seasonal changes in inhibitor β cannot be assumed to reflect changes in ABA only.

The problem of interfering substances in bioassays can be avoided by the use of chemical or physical methods specifically for ABA analysis (see Milborrow (1978a) for a review of the methods for ABA analysis). The availability of such methods, especially gas chromatography (GC), has allowed studies on the seasonal inhibitor β content of buds to be repeated specifically for ABA. However, several studies, in which a specific method for ABA analysis was used, failed to show a correlation between ABA levels and dormancy status of buds. As suggested (Wareing

and Saunders, 1971), it is possible that these results are spurious due to differential losses of ABA during extraction and the necessary extensive purification steps required for the physical methods specific for ABA determinations. However, where corrections for losses were made, no causal relationship between ABA levels and the state of dormancy in buds could be found (Lenton, Perry and Saunders, 1972; Saunders, Harrison and Alvim, 1974; Mielke and Dennis, 1978; Alvim et al., 1978; Dumbroff, Cohen and Webb, 1979; Phillips and Hoffman, 1979). It is possible that a correlation exists but is masked by the extraction of complete or whole buds as opposed to the extraction of bud meristem and leaf primordia separately from the bud scales. More inhibitory activity and ABA per bud was found in extracts of bud scales than in extracts of bud meristems plus primordia of several species, including peach (Dennis and Edgerton, 1961), apricot (Ramsey and Martin, 1970), blackcurrant (Tinklin and Schwabe, 1970), sour cherry (Mielke and Dennis, 1975a,b) and grape (Iwasaki and Weaver, 1977; Emmerson and Powell, 1978). Scale removal from buds caused elongation of the preformed embryonic shoot in maple (Pollock, 1953), beech (Wareing, 1953), rhododendron (Schneider, 1968), sycamore maple (De Maggio and Freeburg, 1969), apple (Abbott, 1969), blackcurrant (Tinklin and Schwabe, 1970) and *Vitis* species (Emmerson and Powell, 1978). It appears that presence of bud scales impose or maintain dormancy of the more central tissues, and therefore, bud scales could function as a supplier of inhibitors or ABA to maintain dormancy. However, this view is not supported by the observation that the ABA content of bud scales of *Vitis* (Iwasaki and Weaver, 1977; Emmerson and Powell, 1978) and sour cherry (Mielke and Dennis, 1975a,b) remained relatively unchanged throughout the winter and during chilling, when the dormancy status of the buds was changing.

A correlation between endogenous ABA levels and the state of dormancy may not be necessary for ABA to have a regulatory role in dormancy. Wareing has often suggested and stressed the importance of a balance between growth promoters and inhibitors in the hormonal control of bud dormancy (Eagles and Wareing, 1964; Wareing, El-Antably, Good and Manuel, 1967; Wareing, 1969, 1978; Wareing and Saunders, 1971). The demonstration that exogenous applications of gibberellic acid (GA_3) can counteract the ABA induced inhibition of stem elongation and bud burst (Eagles and Wareing, 1964; Digby and Wareing, 1964; Thomas,

Wareing and Robinson, 1965; El Antably et al. 1967), and that endogenous gibberellins (GA's) and cytokinins (see sections 1.4.3 and 1.4.4) increased during natural bud burst lend support to this. The concept of endogenous inhibitor-promoter balance predicts that the induction of dormancy involves a shift in the endogenous balance in favour of inhibitors, such that extension growth ceases, and that the release of dormancy involves a shift in the balance in favour of promoters such that extension growth resumes. If ABA is the inhibitor involved in the photoperiodic induction of bud dormancy of tree seedlings, then, as suggested by Lenton, Perry and Saunders (1972), a shift in the balance in favour of inhibition must involve a change in the levels of promoters only. In some instances, however, endogenous ABA levels may also be affected by photoperiod such that a rise in levels occurs under SD's. At the time of bud burst and during chilling, the balance between inhibitors and promoters is shifted in favour of promotion through the increase in promoters (GA's and cytokinins), and in some instances, by an accompanying decrease in inhibitors, especially ABA. Whilst the hypothesis that the regulation of bud dormancy involves an endogenous balance between ABA and growth promoters is plausible and attractive, as yet there is no direct evidence that such a regulatory mechanism is operating during the dormancy cycle of woody species.

The hypothesis that ABA plays an important role in the regulation of natural bud dormancy was based not only on studies on endogenous ABA levels in buds but also on studies on the effects of exogenous ABA applications to buds and shoots of woody species. The application of a crude extract, assumed to contain natural ABA, to leaves of actively growing *Betula pubescens* seedlings (Eagles and Wareing, 1964) and of synthetic ABA to leaves of actively growing *Acer pseudoplatanus*, *Betula pubescens* and *Ribes nigrum* seedlings, induced bud dormancy in these species (El Antably et al., 1967). Extension growth was arrested and dormant buds, with apparently normal bud scales, formed in response to the application treatments, given under conditions suitable for normal growth. This demonstration of the induction of bud dormancy by ABA strongly supported the concept of dormancy regulation by endogenous ABA. Further reports substantiating these findings are, however, scarce. In contrast, there are several reports that are inconsistent with the observation that exogenous applications of synthetic ABA induces dormancy in buds. Hocking and Hillman (1975) studied the effects of

leaf applied synthetic (±) ABA on the growth and dormancy of *Betula pubescens* Ehrh. and *Alnus glutinosa* Gaertn. Under 16 h photoperiods they found that ABA did not induce or promote the formation of resting buds in these species. Radiotracer studies, however, showed that only a trace of the applied radioactivity could be recovered from the apices as unchanged ABA. Perhaps the failure to induce dormant bud formation was due to a lack of ABA reaching the apices. Earlier, El Antably et al. (1967) had found that it was necessary to make supplementary direct applications to the apices in addition to continuous application by leaf immeersion, to induce bud dormancy in birch. Similarly, spraying shoots of field growing apple and pear trees or stem cuttings of several species when placed under conditions conducive to growth, with an ABA solution, did not result in delayed bud burst. However, if the cut bases of the stems were placed in solutions of ABA, bud break was delayed. El Antably et al. (1967) concluded that ABA can induce, prolong or maintain dormancy if enough ABA reaches the buds. However, with a northern ecotype of *Acer rubrum*, Perry and Hellmers (1973) found that ABA, applied exogenously by leaf immersion and by spraying whole plants, inhibited stem elongation but did not cause the formation of dormant buds comparable to those produced by the transfer of untreated plants to dormancy inducing SD's. Growth and normal leaf formation were resumed as soon as ABA application was stopped. Growth of a southern ecotype was only slowed by the application of ABA and no bud formation of any kind was observed. Similarly, Robitaille and Carlson (1971, 1976) found that injections of ABA into trunks of young apple trees, growing under 16 h photoperiods, caused an inhibition of stem extension and at high concentrations caused the formation of terminal buds "characteristic of summer dormancy". Whilst a linear relationship between growth inhibition and the ABA concentration injected was found, the effect of ABA on growth was temporary, since growth resumed almost immediately after injections were terminated. Cathey (1968) also reported that applications of ABA only partially mimicked the growth characteristics induced by SD's in the growth of certain woody ornamentals. Similary, Juntilla (1976, 1978) found that ABA applied directly to the apex or indirectly via a leaf did not induce apical growth cessation and shoot tip abscission in two species of willow, *Salix pendandra* and *Salix caprea*, whereas the exposure of similar plants to SD's did. In another species of willow, *Salix viminalis*, which also

responds to SD's by aborting the growing apices, Saunders et al. (1974) found that the application of a high concentration of ABA inhibited extension growth completely but did not cause the usual SD response. These reports in the literature raise doubts as to the ability of ABA to inhibit apical growth and to mimic or substitute for the effects of SD's in inducing the formation of innately dormant buds.

The reported ability of ABA to maintain or prolong bud dormancy has also been questioned by several reports within the literature. Singha and Powell (1976) reported that trunk injections of ABA during winter led to an increase in the amount of ABA in the buds of apple trees growing under natural conditions, but did not cause a delay in bud break in the following spring. Emmerson and Powell (1978) showed that exogenous applications of ABA failed to inhibit the growth of non-resting, chilled *Vitis* buds. Mielke and Dennis (1974, 1978) reported that applications of ABA to field grown sour cherry trees did not delay bloom of the flower buds. However, under conditions conducive to growth, a delay in bud burst was observed when stem cuttings were placed with their cut bases in solutions of ABA, but this effect decreased as dormancy was broken and only occurred when ABA was applied before visible bud swell. Similarly, McWha and Langer (1979) showed that ABA effectively delayed bud burst in three species of willow (*Salix alba*, *Salix fragilis* and *Salix alba/babylonica*) but its effect diminished as spring approached. More importantly, they found that the number of buds which grew, when cut stems were transferred to long day conditions conducive to growth, was dependent on various factors including the species, the position of the buds on the stem, the presence and absence of leaves, and harvest date. Interactions were also observed between species and harvest dates, bud position and species, and bud position and harvest date. Generally, during the winter, the basal buds grew more readily, but as spring approached there was an increasing tendency for apical buds to grow. The presence of leaves negated the effect of ABA in two (*Salix fragilis* and *Salix alba/babylonica*) of the three species. This is in contrast to Mielke and Dennis (1978) who found no interaction between the effects of ABA and presence of leaves on bud burst in sour cherry. The results of McWha and Langer (1979), however, raise the question as to whether the method of recording the number of apical buds, which commence growth during a standard period, is sufficient when assessing the degree of dormancy possessed by buds of

woody species. Such a method was commonly used in early studies on ABA effects on bud burst (Little and Eidit, 1968; Haissig and King, 1970; El Antably et al., 1967). The literature cited above show that exogenous applications of ABA may not prolong or maintain bud dormancy in woody species.

In addition to the winter buds of temperate woody species, dormant buds are also found on the specialised overwintering organs such as bulbs, tubers, rhizomes and turions. Several studies on the dormancy of these organs have revealed evidence which is suggestive of ABA having a regulatory role in the development and control of bud dormancy in these organs. A decrease in endogenous inhibitor activity as dormancy is broken naturally or artificially has been reported for buds of potato (Hemberg, 1949a, 1952, 1954, 1958b; Steward and Caplin, 1952; Blommaert, 1954; Varga and Ferenczy, 1956, 1957a). The observations made by Hemberg formed the basis of his postulation that growth inhibiting substances were of importance in the regulation of the dormancy of buds. Abscisic acid or abscisic acid-like inhibitors have been reported to decrease as dormancy is broken in onion (Thomas, 1969); dutch iris bulbs (Tsukamoto and Ando, 1973), tulip bulbs (Syrtanova, 1974), gladiolus corms (Tsukamoto and Konoshima, 1972; Ginzburg, 1973; Konoshima and Tsukamoto, 1978); freesia corms (Masuda and Asahari, 1978) and purple nutsedge tubers (Teo, Nishimoto and Tang, 1974). Applications of synthetic ABA inhibited bud or sprouting in gladiolus cormels (Ginzburg, 1973), purple nutsedge tubers (Teo and Nishimoto, 1973) and in *Begonia*, but not *Dioscorea* tubers (Hashimoto and Tamura, 1969a). Although early attempts (Hemberg, 1949a; Buch and Smith, 1959) to reinstate dormancy in potato buds with inhibitor β were unsuccessful, later attempts did succeed (Blumenthal-Goldschmidt and Rappaport, 1965). Similarly, attempts with synthetic ABA to reinstate or prolong bud dormancy in potato tubers were successful (Madison and Rappaport, 1968; El-Antably et al., 1967; Vanes and Hartman, 1969).

Of the specialised overwintering organs, studies on the production of turions in the Lemnaceae (aquatic angiosperms) provides the most convincing evidence that ABA induces dormancy. Under aseptic culture conditions suitable for growth, the addition of low concentrations of ABA to the medium causes not only an inhibition of growth but also the production of turions in *Spirodella polyrrhiza*

(Perry and Byrne, 1969; Van Staden and Bormann, 1969; Saks, Negbi and Ilan, 1980), *Lemna minor* (Van Overbeek, 1968; Van Overbeek and Mason, 1968), *Lemna polyrrhiza* (Stewart, 1969) and *Ultricularia vulgaris* (Winston and Gorham, 1979a,b). Recently, Saks et al. (1980) demonstrated that endogenous ABA is involved in the regulation of the onset of dormancy in *Spirodella polyrrhiza*. It was shown, that at the time of turion formation, *Spirodella polyrrhiza* releases endogenous ABA into the surrounding medium. In water milfoil (*Myriophyllum verticillatum*), however, synthetic ABA induced turion production only under marginally inductive conditions (Weber and Nooden, 1976). Interestingly, a decrease in inhibitor content of turions was found as dormancy of the turions was broken, but there was no decrease in the ABA content of turions. The above studies on specialised overwintering structures provide some supportive evidence of abscisic acid's role in the regulation of bud dormancy of plant species.

Most of the research effort on growth inhibitors that has been considered as being correlated with dormancy has centered around the inhibitor β complex and abscisic acid. Historically, however, the first growth inhibitor that was positively identified and implicated in bud dormancy was the phenolic compound 5,7,4'trihydroxyflavone or naringenin (Hendershott and Walker, 1959a,b). These workers reported the occurrence of naringenin in dormant peach buds and found that the decrease in the naringenin content of buds was correlated with the termination of dormancy. This observation, along with the identification of several phenolic compounds in the inhibitor β fraction of various tissues (Varga, 1957; Varga and Ferenczy, 1957b; Bentley, 1958; Lane and Bailey, 1964), led to the view that phenolic growth inhibitors may have an important role in the regulation of bud dormancy. There exists, within the literature, some reports suggestive of such a role. The occurrence of naringenin in peach flower buds was confirmed by Dennis and Edgerton (1961) and Corgan (1965), but these investigators failed to correlate its changing presence with the dormancy period. Dennis and Edgerton (1961) suggested that because the results were expressed on a fresh weight basis, the decrease in inhibitor content observed by Hendershott and Walker (1959a,b) was not absolute but merely represented a dilution due to swelling of the buds at the time of bud burst. Samish and Lavee (1962) disagreed with Dennis and Edgerton (1961) and confirmed, that on a fresh weight basis or on the

amount per bud, the level of naringenin did fall with the termination of dormancy. However, Corgan (1965) was unable to find a correlation although he did report a similar sharp increase in the naringenin content of buds as they entered dormancy. Prunin, the glycoside of naringenin, has also been reported to occur in peach flower buds (Corgan, 1967; Erez and Lavee, 1969). Erez and Lavee (1969) found that as naringenin accumulated in the buds during autumn and early winter, there was a sharp decrease in prunin. During the period of bud burst, both naringenin and prunin decreased in the buds. Thus, Erez and Lavee (1969) have suggested that prunin, either alone or in combination with naringenin, acted as a causal inhibitor of growth. There are, however, no reports of naringenin or prunin being able to induce dormancy when applied exogenously to growing plants, although Phillips (1962) found an antagonistic effect of naringenin upon the dormancy breaking effect of gibberellins after simultaneous application to peach buds. However, Dennis and Edgerton (1961) reported that exogenously applied naringenin did not delay bud burst in peach. It appears, that naringenin's role in peach bud dormancy is not one of causation or regulation. Altree-Williams, Howden, Keegan, Malcolm and Wyllie (1975) identified several other growth inhibitors of a phenolic nature, in peach flower buds. The significance of these phenolic type compounds in dormancy is not known. Polyphenol oxidase activity in peach buds has been correlated with the decrease in the inhibitory activity of phenolics, and it was suggested (Kenis and Edelman, 1976) that this is an important event in the sequence of phenomena which lead to dormancy release.

In addition to the dormancy of peach, phenolic compounds have also been implicated in the dormancy of other species. Lane and Bailey (1964) in an attempt to characterise the inhibitor β fraction from dormant buds of silver maple (*Acer saccharinum* L.), concluded that it was a phenolic compound on the basis of R_F values and coupling reactions with diazotised reagents. Kononenko, Popravka and Vul'fson (1975) found that the flavonoid aglycone content of birch (*Betula verrucosa*) buds is maximal during the dormant period. Abscisic acid levels do not correlate well with dormancy in yam (*Diosconea batatas*) bulbils but three neutral inhibitors termed "Batatasins" do (Hashimoto, Hasegawa and Kawarada, 1972; Hasegawa and Hashimoto, 1973, 1974). Dormancy breaking treatments resulted in a decrease in the endogenous batatasin levels and exogenous applications of batatasins caused the induction

of dormancy. Two of the compounds appear to be phenols (Hashimoto et al., 1972, 1974). Phenolic compounds have also been implicated in the dormancy of gladiolus corms (Tsukamoto and Ando, 1973; Tsukamoto and Konoshima, 1972; Konishima, Yazawa and Taukamoto, 1973). Similarly, the dormancy of the liverwort *Lunularia cruciata* does not appear to be controlled by abscisic acid but by a phenolic acid, called lunularic acid (Valio, Burdon and Schwabe, 1969; Schwabe and Valio, 1970; Valio and Schwabe, 1970). In contrast to the reports of successful induction of dormancy by phenolic compounds with batatasins in yams and lunularic acid in liverworts, there are no known reports of phenolic acids inducing dormancy in buds of woody plants. Such evidence is vital if phenolics are to be considered as causal agents of bud dormancy.

Recently, another class of growth inhibitor compounds, short chain fatty acids, have also been suggested as being involved in the regulation of dormancy, especially of oats (Berrie, Don, Buller, Alam and Parker, 1975; Berrie, Buller, Don and Parker, 1979). It was reported by this group that dormant oat seeds and buds of sycamore and spruce contain very high levels of short chain fatty acids relative to actively growing tissues. Tsukamoto and Ando (1973) had earlier suggested that short chain fatty acids have an inhibitory role in dormant buds of bulbous plants. Capric acid (C10), a fatty acid with a chain length of nine carbons, was isolated from Dutch Iris (*Iris hollandica*) bulbs and identified by nuclear magnetic resonance, infra red spectroscopy and mass spectrometry (Ando and Tsukamoto, 1974). Endogenous levels of capric acid were well correlated with the degree of dormancy possessed by the bulbs. Short chain fatty acids have been reported (Berrie et al., 1975; Ando and Tsukamoto, 1981) to possess similar chromatographic properties to inhibitor β, and therefore, they may contribute to the inhibitory activity of this fraction. However, it remains to be proven whether the occurrence of short chain fatty acids, and phenolics, in the inhibitor β fraction of woody plants contributes to dormancy regulation. As yet, the ability of short chain fatty acids to induce dormancy does not appear to have been tested for buds of woody species.

In reviewing the literature it is clear that there is considerable and varied evidence to support the inhibitor theory of dormancy regulation. The role of inhibitors in the development of

dormant buds is not clear, but ABA appears to be one inhibitor that is involved in at least some species. Other inhibitors may function in other species. Evidence suggests that ABA or ABA-like activity may increase in buds during the development of dormancy and dormant structures, and decrease during the termination of dormancy. However, the contradiction raised by Lenton, Perry and Saunders (1972) has not been resolved, nor has the failure of ABA and other inhibitors to reinstate dormancy and prevent outgrowth of the embryonic shoot. Such inconsistencies fail to support an unambiguous involvement of ABA or inhibitor β in bud dormancy of woody species. It is possible that abscisic acid's role in dormancy is complicated by its interaction with growth promoters.

1.4.2 Auxin

The now classical studies on the response of the oat coleoptile to light led to the discovery of auxin and the recognition of the theory that plant growth was under hormonal control [for a detailed historical account refer to Boysen-Jensen (1936) and Went and Thimann (1937)]. Subsequent research on the role of auxin in growth processes was initiated and developed under the influence of two dicta: "Without auxin no growth" and "when there is little auxin there is little growth, when more auxin more growth". These two dicta, first enunciated by Went (1928), led to the suggestion by Boysen-Jensen (1936) that bud dormancy was due to a lack of auxin or auxin precursors. However, a second thought was that dormancy was caused by a supra-optimal concentration of auxin in the buds. This theory was based on the observations of Thimann and Skoog (1934) and Van Overbeek (1938) that auxin inhibited the outgrowth of lateral buds on a plant stem. Auxin, then, was thought of as a promoter and as an inhibitor of growth.

These two concepts of dormancy led to numerous attempts to measure the content of auxin in tree buds at different times during the year, in order to correlate the ability of buds to burst with their auxin content. The first reported studies on the auxin content of tree buds were made by Zimmermann (1936) and Avery, Burkholder and Creighton (1937), both of whom used diffusive techniques to extract auxin and the *Avena* curvature test to quantify it. In both studies no auxin was found in the buds during late winter, but auxin was detected in sprouting buds. However the studies used buds in the

quiescent state, and therefore, it was not proven that dormancy was a result of a deficiency in auxin. On the other hand, Bennett and Skoog (1938), in a study on the auxin content of pear and cherry buds, did include buds in the innate dormancy state. No auxin or auxin precursors were detected in the buds until late winter when dormancy had been broken. According to Bennett and Skoog (1938) the occurrence of auxin is correlated with the breaking of dormancy, and hence auxin was acting as a promoter of growth. However, similar reports from several other investigators (for references and reviews see Samish, 1954; Hemberg, 1965) contained widely divergent results regarding the occurrence and levels of auxin in tree buds during the seasons. A possible explanation for the discrepancy is that different techniques were employed to extract auxin. Early auxin workers used diffusion techniques to extract, whereas the later workers relied on solvent extraction methods. However, such an explanation is not wholly satisfactory in view of some discrepancies. For example, Kassem (1944, cited in Eggert, 1953) determined the auxin content of ether extracts of pear buds and found the content fell towards the end of dormancy. Therefore, he supported the view that dormancy was caused by a supraoptimal content of auxin in the buds and that growth could only commence after the auxin content had fallen to some other lower level. Eggert (1953) also supported the supraoptimal theory. He made an aqueous extract of apple buds, by boiling the tissue in IN NaOH, and found that the auxin content was higher during the dormant phase than after this stage. On the other hand, this view was not supported by Blommaert (1955) who extracted peach buds with ether and fractionated the extract by means of paper chromatography. He found that the auxin content of the buds fell whilst they were entering dormancy. No ether extractable auxin was detected in dormant buds during the winter, but with the onset of bud break in the spring, the auxin content in the buds rose again and continued to remain high during the vegetative growth period until the onset of dormancy. The controversy surrounding diffusible and extractable auxin was not resolved and the failure to demonstrate any consistent correlations in support of either hypothesis, together with the emergence of the inhibitor concept of dormancy, led to disinterest in the idea that auxins are responsible for the control of bud dormancy. Nevertheless, through the advent of paper chromatography and the use of various bioassays, many investigators were able to assay auxin activity as well as the inhibitor content of extracts of buds harvested at

various times during the year.

The findings of Waxman (1957), Phillips and Wareing (1958), Dorffling (1963a,b), Eliasson (1969), Zaerr (1967), Langrova and Sladky (1971) and Alden (1971) suggested that the development of dormancy was accompanied by decreased auxin levels or production. Similarly, dormancy-inducing SD's caused a reduction in the auxin activity of extracts of sumac apices (Nitsch, 1957a), *Populus canadensis* apices (Nitsch, 1963) and of blackcurrant leaves and buds (Kuzina, 1970). The auxin levels and production appeared to be low during the dormant phase but increased as dormancy was terminated, as demonstrated in terminal buds of sycamore (Phillips and Wareing, 1958) and *Pinus palustris* (Allen, 1960). Auxin determinations in all these reports were based on bioassays or colorimetric determinations of crude extracts, and therefore, are subject to the same problems as the measurement of inhibitors, especially that of interfering substances. More recent studies using modern physical methods for indole-3-acetic acid (IAA) determinations are scarce. Alden (1971) confirmed the presence of IAA in buds of Scots pine (*Pinus silvestris*) by fluorescence spectrophotometry and gas chromatography, but used the *Avena* straight growth test to detect and determine the seasonal variation in IAA content of buds. He found high levels of auxin in buds during their vegetative growth phase, but no auxin during their dormant phase. Emergence from dormancy coincided with the reappearance of auxin in the buds. Similarly, Zabkiewicz and Steel (1974) confirmed the presence of IAA in extracts of *Pinus radiata* buds by GC-MS, but used a bioassay method to determine the seasonal variation in the level of auxins and inhibitors. They found more auxin-like activity during the spring and summer than in winter. It should be noted, however, that *Pinus radiata* did not show a period of bud dormancy during the winter although growth was slower than in the spring. Other investigators have concentrated on the determination of IAA, by modern methods, in shoots and other tissues of trees rather than on buds. In *Picea abies*, the maximum IAA level occurred in shoots at the time of maximum shoot elongation and then fell rapidly (Dunberg, 1976). DeYoe and Zaerr (1976), using GC-MS, demonstrated the occurrence of IAA in Douglas fir shoots and found that winter shoots had little or no IAA compared with spring shoots. The occurrence and determination of IAA levels in growing shoots of Douglas fir was confirmed by Caruso, Smith, Smith, Cheng and Daves (1978), and

Weiler and Ziegler (1981) showed the occurrence of IAA in the phloem exudates of several tree species. The occurrence of IAA in the cambial region of trees, and the possible role of IAA in the regulation of seasonal cambial activity, have been investigated by Jenkins and Shepherd (1974) and more recently by Wodzicki (1978), Wodzicki and Wodzicki (1980), Little, Heald and Browning (1978), Little (1981), and Little and Wareing (1981). A positive correlation between cambial activity and IAA levels or IAA metabolism was not observed. The metabolism of exogenously applied IAA in woody tissues (xylem) has been studied, but the occurrence and role of endogenous bound IAA has yet to be determined (Nix and Wodzicki, 1974; Riov and Gottlieb, 1980).

The evidence, then, indicates that high auxin activity in buds and shoots is associated with the periods of active extension growth in the spring and summer, and low auxin levels with periods of low growth activity and dormancy in the autumn and winter. Bud burst is coincident with increasing auxin levels in the buds. Whilst this seasonal variation in auxin content of buds is suggestive of a role in dormancy, a causal relationship between the appearance of auxin in dormant buds and the emergence from dormancy is not conclusive. The exogenous application of auxin does not promote bud burst in many species, including sycamore (Wareing, 1965), apple (Pieniazek and Jankiewicz, 1967), rhododendron (Schneider, 1970), peach (Marth, Havis and Batjer, 1947) and grape (Nogond, 1957; Weaver, McCune and Coombe, 1961). Further reports on the lack of a promotive effect on bud break, or in some cases retardation of bud break by exogenous auxin, are given by Doorenbos (1953). In contrast, only a few reports claim a promotory effect on bud burst by auxin. Amlong and Naundorff (1938) obtained a promotion of bud burst in *Syringia vulgaris* after repeated applications of IAA or NAA. Similar results were also reported by Bennett and Skoog (1938) and Mitchell and Cullinan (1942), who studied floral and vegetative buds of peach and pear. From these studies, and the inconclusive evidence of endogenous auxin levels during bud break, the role of auxin during the outgrowth of buds is unclear.

The lack of any recent research on the role of auxin or IAA in bud dormancy may be an indication of the dismissal of auxin as a major factor in its regulation. However, the involvement of IAA in many aspects of plant development (see Letham, Goodwin and Higgins, 1978) suggests IAA may have some involvement in dormancy.

1.4.3 Gibberellins

The discovery of gibberellins (GA's) in plant tissues by Japanese workers and the determination of the structure and synthesis of gibberellic acid (GA_3) led to initial experiments in which the effects of GA_3 on plants were determined. [For a historical account see Stowe and Yamaki (1957, 1959), Stowe, Stodola, Hayashi and Brian (1961).] It was observed that exogenous applications of GA_3 promoted many of the growth and development processes of plants, and therefore, it was suggested that GA's might function as endogenous hormones in plants (Radley, 1956; Phinney, West, Ritzel and Neely, 1957).

The effect of exogenous applications of GA_3 to tree buds and seedlings was widely studied and the evidence from such studies suggested that GA's may have a role in the regulation of dormancy. Gibberellic acid has been reported to block the photoperiodic induction of bud dormancy under controlled conditions (Lockhart and Bonner, 1957; Nitsch, 1957b; Juntilla, 1976) and under natural conditions (see Romberger, 1963; Vegis, 1964, 1965). In *Salix pentandra*, the cessation of apical growth under natural SD's or artifically induced conditions was significantly delayed by a single GA_3 application (Juntilla, 1976). Other reports indicate that GA_3 can break the dormancy of tree buds of several species (Marth, Audia and Mitchell, 1956; Romberger, 1963; Vegis, 1964; Wareing and Saunders, 1971), including peach (Donoho and Walker, 1957; Walker and Donoho, 1959), cranberry (Rigby and Dana, 1972) and a hybrid of birch (Vlasov et al., 1978). Although these reports indicate that GA's can maintain and/or stimulate dramatic shoot extension and break bud dormancy in a wide range of tree species, there are species which respond differently. For example, in peach, Hatch and Walker (1969) found that GA_3 broke dormancy of leaf buds but not flower buds. In conifers, Lockhart and Bonner (1957) found that GA_3 did not break bud dormancy when plants were kept under SD conditions. Similar results were found for apple buds (Hull and Lewis, 1959), large-leaved linden (*Tilia platyphyllos*) buds (Lyr, Hoffmann and Richter, 1970) and excised rhododendron flower buds (Schneider, 1970). In several woody species, GA_3 is able to stimulate bud outgrowth only before or after but not during the phase of deep dormancy (Leike, 1967; Paiva and Robitaille, 1978; Wood and Hanover, 1981). The response of buds to GA_3, then, varies according to the degree of dormancy or chilling requirement possessed by the buds. Indeed, buds of several woody

species are more responsive to GA's following a cold treatment (Marth et al., 1956; Donoho and Walker, 1957; Lasson, 1960; Lyr et al., 1970). However some reports indicate that GA treatments actually prolong bud dormancy when applied to the buds, e.g. in grapes (Weaver, 1959; Iwasaki, 1980). Similarly, the application of GA_3 to peach (Corgan and Widmoyer, 1971; Edgerton, 1966; Proebstring and Mills, 1964; Bottini, Goleniowski and Correa, 1978; Bowen and Derickson, 1978), grapes (Alleweldt, 1959; Rives and Pouget, 1959), *Prunus avium* (Brian, Petty and Richmond, 1959) and several *Prunus* species (Bradley and Crane, 1960) in late summer or autumn, before leaf fall, delayed bud break the following spring.

It is not clear how GA's act to break or reinforce dormancy. In blackcurrant buds (El Antably et al., 1967) and aseptically cultured buds of Norway maple (De Maggio and Freeburg, 1969), GA_3 overcomes the inhibitory effect of ABA on bud growth. Gibberellic acid has also been shown to counter competitively the inhibitory effect of naringenin on the growth of peach buds (Phillips, 1962), and was able to offset the inhibitory effect of inhibitor β on birch buds (Eagles and Wareing, 1964). Similarly, in birch, the stimulatory effect of GA_3 on bud burst was depressed by abscisic acid when jointly applied to cuttings (Vlasov et al., 1978). This evidence suggests that GA and inhibitors may interact competitively in the plant. Whether GA's cause a decrease in the inhibitor content as it breaks bud dormancy is, as yet, undetermined. In the case of peach flower buds, where GA_3 application delayed bud burst when applied to the foliage during autumn, the concentration of abscisic acid in buds was decreased in midwinter compared with untreated buds (Bowen and Derickson, 1978).

Whilst the main thrust for the implication of GA's in dormancy regulation has come from studies on the exogenous effects of GA_3, there are some studies on endogenous gibberellins in buds. However, most of these studies have involved the use of bioassays to detect and determine the GA-like activity of bud extracts, and therefore, the results are open to interpretation and may be misleading. For example, in crudely fractionated extracts, inhibitory substances, that are difficult to separate from GA's by chromatographic methods, may mask the GA effect on the bioassay material (Bailiss and Hill, 1971). Nevertheless, from such studies a seasonal pattern of GA activity in buds and other tissues has emerged. Gibberellin-like activity has been reported to increase

before bud burst or as buds emerge from dormancy in sycamore (Eagles and Wareing, 1964), birch and blackcurrant (Tumanov, Kuzina and Karnikova, 1970), cranberry (Eady and Eaton, 1972), Douglas fir (Lavender, Sweet, Zaern and Hermann, 1973), Damask rose (Koseva and Decheva, 1976), and persimmon (Wurzburger and Farkash, 1976). Similarly, in a study on the physiology of dormancy release in buds of *Populus balsamifera*, Bachelard and Wightman (1974) found that the GA activity of buds does not increase until dormancy is broken, and then decreases before rising again to a peak which coincides with bud burst. In *Picea abies* (Norway spruce) shoots, the GA-like activity was greatest at the time of maximum shoot elongation after dormancy release (Dunberg, 1976). Dathe et al. (1978) reported that there was no gibberellin activity in birch tree sap collected in the winter, whereas in the bleeding sap, in the spring, high levels of gibberellin-like activity were detected. Studies on endogenous levels of GA's in buds and other tissues, during the transition into the dormant state, indicate that the level of GA's falls. For example, in terminal buds of walnut (*Juglans regia*) the GA activity decreases well before the development of dormant buds (Langrova and Sladky, 1971). Dormancy-inducing SD's caused a decrease in the GA activity in birch (*Betula pubescens*) stem tissue and in willow (*Salix viminalis*) phloem sap (Hoad and Bowen, 1968). From these few studies on endogenous GA's, it appeared GA's are absent in dormant buds and that endogenous levels increase in buds and other tissues once dormancy is broken. The endogenous levels remained high in actively growing shoots until the onset of dormancy. Dormancy, however, was not thought to be the result of a lack of GA's although their presence could delay the onset of dormancy. Instead, the strong attraction of the inhibitor hypothesis and the demonstration of a reciprocal variation between levels of GA-like activity and levels of ABA or unidentified inhibitors of the fraction in extracts of buds, led to the concept that dormancy was a consequence of high ABA and low gibberellin levels in the buds, whereas low ABA and high gibberellin levels result in the release from dormancy (Eagles and Wareing, 1964; Wareing, 1969; Wareing and Saunders, 1971; Bachelard and Weightman, 1974). This concept of dormancy as a consequence of an unfavourable balance between promoters and inhibitors, however, is based on bioassay data. Where extracts contain a mixture of promoters and inhibitors, the reciprocal variations observed can be

explained by variations in the concentration of either promoters or inhibitors and not necessarily a change in both. The results of Ramsay and Martin (1970a), who found a reciprocal variation between inhibitors and GA-like activity in apricot buds but in a reverse, and therefore, anomalous manner to the hormonal balance concept of dormancy, may well be explained on this basis. Therefore, until specific information on endogenous gibberellins of buds is obtained, by physical or chemical analysis, the hormonal balance concept of dormancy is premature.

The study of gibberellins, however, is greatly complicated by the existence of a large number of different gibberellins [57 listed by Bearder (1980)] and the uncertainty over which are functioning as hormones. The identification of which gibberellins exist in buds has only recently been attempted. Lorenzi, Horgan and Heald (1975) identified *iso*-GA_9 and the glucoside of GA_3 in Sitka spruce (*Picea sitchensis*) needles, and these gibberellins also appear to be present in the buds. This study, which relied on bioassays for quantification, revealed that, in the buds after dormancy is broken, the more polar GA activity shows no change or a slight decrease whereas the less polar activity increases. In the needles, the polar and less polar GA's both increased after dormancy was broken, the less polar gibberellins increasing later than the more polar gibberellins. At the time of dormancy development, the gibberellin activity of the needles increased but as the buds entered dormancy the amount of less polar GA-like activity dropped, whereas the more polar GA activity was remained unchanged at a high level. Further work on identification of the polar and non polar gibberellins is required before seasonal variations in the levels of specific gibberellins in extracts is established. The existence of several gibberellins in buds has also been demonstrated in peach flower buds (Bottini, Bottini and Correa, 1976). During the dormancy of the flower buds, gibberellin-like substances increased in a select way, with the less polar gibberellins increasing during dormancy and the more polar gibberellins increasing near the time of bloom. The evidence from the above two studies indicates that endogenous gibberellins may show a differential pattern of change as dormancy is broken or induced without any corresponding change in overall gibberellin activity or level, and hence the identification of all gibberellins in the extracts is important.

Not all gibberellins have the capacity to break dormancy. Photoperiod-induced dormancy in *Weigla* could be broken by GA_1 and GA_3, but not GA_2 and GA_4 (Bukovac and Wittwer, 1961). Both GA_7 and GA_3 were capable of breaking dormancy of sugar maple buds, but GA_7 tended to be more active than GA_3 (Wood and Hanover, 1981). These observations suggest that not all the gibberellins function equally in dormancy, and highlights the need to analyse which gibberellins are active internally in a plant at different times of the year. That gibberellin metabolism, and specifically interconversions between various gibberellins, can change in woody tissues is illustrated by a study of the metabolism of GA_4 by shoots of Douglas fir (Wample, Durley and Pharis, 1975). The ability of Douglas fir shoots to metabolise GA's has been shown to depend on whether the shoot was in a condition of bud set, bud break or bud elongation. At all times, the relatively nonpolar GA_4 was rapidly metabolised to the more polar gibberellins, including GA_{34} and GA_2, but there were quantitative differences in metabolism depending on the growth state of the shoot.

Through the use of methods such as GC-MS, specific information on the identity of the endogenous gibberellins in buds of each species at various times of the year should be realised and with such information, a concept of dormancy implicating gibberellins can be proposed.

1.4.4 Cytokinins

The term cytokinins was suggested by Skoog, Strong and Miller (1965) to describe compounds with cell division promoting activities. The first such compound isolated and identified from a natural source was kinetin (K) (Miller, Skoog, Okumura, von Saltza and Strong, 1955, 1956). However, kinetin itself is not a natural compound, but a synthetic compound derived from autoclaved DNA of herring sperm. Since the discovery of kinetin, naturally occurring substances such as zeatin (Z), zeatin riboside (ZR), isopentenyl adenine and isopentenyl adenosine, and synthetic compounds, such as benzyl adenine (BA) and SD 8339, have been shown to possess cytokinin-like regulatory properties. Besides having cell division and cell enlargement properties, cytokinins were shown to overcome correlative inhibition of lateral buds (Sachs and Thimann, 1967) and release buds from dormancy in many species.

Exogenous applications of cytokinins break dormancy in apple buds (Chvojka, Travnicek and Zakourilova, 1962; Pieniazek, 1964a; Pieniazek and Jankiewicz, 1967; Jones, 1967; Kender and Carpenter, 1972), Monterey Pine (*Pinus radiata*) buds (Kummerow and de Hoffmann, 1963), grape buds (Weaver, 1963), citrus buds (Cooper, Young and Henry, 1969), tea crab-apple buds (Broome and Zimmerman, 1976) and peach buds (Weinberger, 1969). In comparing the effects of cytokinins and gibberellins on dormancy release, it was found that both growth regulators are capable of breaking dormancy in many species, but in specific cases, such as apple, only cytokinins are effective. Following bud break, cytokinins are not capable of stimulating stem elongation unless gibberellins are also present (Williams and Billingsley, 1970). Cytokinins, like gibberellins, do not act with equal effect at all stages of dormancy, e.g. dormant flower buds of coffee will not respond to kinetin unless the trees have been given a partial dormancy breaking dry spell (Van der Veen, 1968). On the other hand, less drought is required for GA_3 to break dormancy (Browning, Hoad and Gaskin, 1970). Other studies also suggest that the response of buds to cytokinins varies according to the degree of dormancy within the buds. Leike (1967) reported that kinetin, and GA_3, induced outgrowth of buds of several woody species but only during the pre- and post dormancy phases and not during the intervening period when the chilling requirement of the buds had, presumably, not been fulfilled. Lessening the chilling required by partial cold treatment did enhance the effect of kinetin on release from dormancy in apple buds (Pieniazek, 1964a). Similarly, in peach the synthetic cytokinin SD 8339 was able to break dormancy after the chilling requirement had been partially fulfilled (Weinberger, 1969). This was in contrast to gibberellic acid which could break the dormancy of unchilled buds. In aseptically cultured explants of citrus buds, benzyl adenine promoted or retarded outgrowth depending on when, during the seasonal cycle, the buds were transferred to the benzyl adenine containing medium (Altman and Goren, 1974). In sugar maple, benzyl adenine was incapable of breaking dormancy (Wood and Hanover, 1981), and Broome and Zimmerman (1976) found that only some cytokinins they tested were effective in stimulating bud break. Similarly, in an *in vitro* bud culture study (Singha and Powell, 1979), using non-dormant vegetative axillary bud explants obtained from apple, the inhibition of bud burst by purine analogues was only partially overcome by BA.

In another *in vitro* study (Pieniazek, 1968) using apple shoot tip explants, BA was capable of breaking dormancy of axillary buds but not apical buds. However, other *in vitro* studies with apple buds (Borkowska and Powell, 1979; Borkowska, 1980a,b) revealed that single bud explants break dormancy merely as a result of excision from the tree, irrespective of the presence or absence of BA or the state of the chilling requirement of the mother tree. Continued development of buds from chilled or unchilled trees was, however, markedly different and BA was found to stimulate stem elongation in the unchilled developing explants. Jones (1967) also reported stimulated stem elongation and leaf production in isolated apple shoots. Despite some anomalies, the results from the above studies, in which synthetic cytokinins were exogenously applied to buds, suggest that endogenous cytokinins may have a role in dormancy or at least some involvement in the activation of dormant buds.

Several studies have been made on the changes that take place in endogenous cytokinin activity of buds and other tissues of woody plants during chilling and bud burst. Bioassays were used in these studies to detect and assess the endogenous cytokinin activity. Domanski and Kozlowoski (1968) reported an increase in cytokinin-like activity in buds of excised twigs of *Populus balsamifera* and *Betula papyrifera* after growth had been induced by hot water-bath treatment. In *Populus tremula* and *Acer platanoides* buds, Englebrecht (1971) found that the cytokinin activity, detected by the tobacco callus bioassay, increased during the natural termination of dormancy. The cytokinin activity had chromatographic properties similar to zeatin. Hewett and Wareing (1973a,b) studied changes in cytokinins occurring in *Populus* x *robusta* and *Acer pseudoplatanus* buds during chilling and bud burst. They used the soybean callus bioassay and found that dormant buds contained no detectable cytokinin activity, but a sharp rise in activity occurred just before bud swelling. After bud swelling, the cytokinin content dropped sharply. Chromatography on a Sephadex LH-20 column indicated that at least five cytokinins were present in the buds. The predominant ones had similar elution volumes to zeatin and zeatin riboside. In a study on the dormancy of sugar maple seedlings, Taylor and Dumbroff (1975) exposed dormant seedlings to dormancy breaking low temperatures and found that the cytokinin activity in buds, as detected by the cucumber cotyledon bioassay,

decreased until the chilling requirement was fulfilled and then increased sharply. Paper chromatography (isopropanol/NH_3/H_2O solvent) revealed three cytokinins were present, with the most active cytokinin being situated at R_F 0.9. Further studies (Dumbroff and Brown, 1976) revealed that the increase in cytokinin activity coincided with the renewal of root growth, and it was suggested that the roots may have been the source of the cytokinins. Borkowska (1976), in a study of the content of endogenous cytokinins in apple buds during dormancy and swelling, found that the occurrence of cytokinin-like compounds was dependent on the stage of dormancy and position of the buds on the shoots. Cytokinin activity, detected by the amaranthus and soybean callus bioassays, was high when buds started to swell and was very low before actual bud burst. At all times during dormancy, the cytokinin content was higher in the apical buds and buds situated near the apex than in other buds. A complex of cytokinins was detected in the chromatographed (paper, butanol/HAc/H_2O, 12/3/5) extracts, but the cytokinin at R_F 0.6 - 0.88 showed the highest activity than the rest and this cytokinin appeared in all buds at all stages of dormancy. Van Staden (1979) determined the changes in the cytokinin levels of excised buds of *Salix babylonica* cultured aseptically, and found that the buds had increased cytokinin levels after 15 days of culture, although initially, the cytokinin levels had decreased. Most buds had become swollen and some were showing signs of bursting. Most of the cytokinin increase, which was detected by the soybean callus bioassay, co-chromatographed with zeatin glucoside, but zeatin riboside and zeatin were also present. From the study, Van Staden concluded that the buds did not have the capacity to synthesise to cytokinins but the callus derived from the exposed cambial region of excised buds was responsible for the increased levels. Henson and Wareing (1977) also reported that endogenous cytokinin activity in *Xanthium strumarium* buds declined in the first 24 hours after detachment from the plant. These studies on endogenous cytokinins in buds suggest that cytokinins increase at the time of bud swell and bud burst, but the buds themselves may not be capable of synthesising their own cytokinins. The roots of plants have been suggested (Skene, 1975) as being a source of cytokinins occurring in the shoots.

The occurrence of cytokinins in the xylem sap has been demonstrated in several woody species. Skene (1975) has listed the

species and emphasised that the nature and properties of cytokinins present in xylem saps can be altered by a wide range of environmental and plant factors, including root temperature, the nature and pH of the root medium, the stress to which plants are exposed, photoperiod and plant age. Nevertheless, an association between dormancy release and cytokinin activity in the xylem saps has been observed in several woody species, including apple (Luckwill and Whyte, 1968), grapes (Skene, 1972), *Populus* x *robusta* (Hewett and Wareing, 1973a,b) and *Salix viminalis* (Alvim et al., 1976). Several studies (Skene, 1972; Hewett and Wareing, 1974; Alvim et al., 1976; and those listed by Skene, 1975) indicate that zeatin and zeatin riboside are common components of xylem saps, and that the levels of these cytokinins increased as dormancy was broken. However, Purse, Horgan, Horgan and Wareing (1976) found that spring sap from sycamore maple did not show significantly greater quantities of cytokinins just before or during bud break. Therefore, the significance of the cytokinins found in the sap of trees, during the spring, may or may not be related to dormancy release.

Information on the cytokinin content of buds during the onset of dormancy is sparse, although in two recent studies, Horgan and Wareing (1980) and Darall and Wareing (1981), the cytokinin content of leaves and roots during dormancy induction were studied. In these studies, it was found that dormancy-inducing nitrogen deficiency in *Acer pseudoplatanus* caused only a small reduction in the cytokinin-like content of leaves and roots. In birch, *Betula pendula*, nitrogen deficiency did not induce dormancy, but the leaves and roots showed a greater reduction in their cytokinin content than corresponding *Acer pseudoplatanus* tissue. Unfortunately, it was not determined whether any changes in the cytokinin content of buds had occurred during the nitrogen deficiency treatment. In *Xanthium strumarium*, an annual plant, the metabolism of exogenously supplied zeatin to zeatin riboside in detached buds was not affected by photoperiod since no difference in either the rate or pattern of zeatin metabolism was found (Henson and Wareing, 1977). It remains to be seen whether or not a change in the cytokinin activity of buds of woody species occurs in response to photoperiod.

There are some reports on the cytokinin content of leaves during the natural onset of dormancy and at other times during the growing

season. Englebrecht (1971) found a decrease in the cytokinin activity of sycamore maple and aspen leaves during the late summer - early autumn period. The cytokinin activity had a similar R_F to zeatin, although a zeatin-mononucleotide-like substance was also present in the leaves, and its content rose as autumn progressed. In *Ginkgo biloba* and *Salix babylonica*, Van Staden (1976a,b, 1977) found changes in cytokinin activity as leaves aged, and in these species the total cytokinin activity increased with leaf age. Qualitative differences in cytokinins also occurred. In mature *Ginkgo* leaves, zeatin glucoside and zeatin riboside glucoside were the major cytokinins present, whereas in young leaves zeatin and zeatin riboside were the predominant cytokinins present. In mature, senescing *Salix babylonica* leaves, the major cytokinin was zeatin glucoside (Van Staden, 1976a). The increase in cytokinin activity of *Gingko* and *Salix* leaves with ageing is in contrast to that reported for *Populus* x *robusta* leaves by Hewett and Wareing (1973b). They reported that the number and levels of cytokinins decreased during the season, and with leaf age, such that yellow senescent leaves had only one detectable cytokinin, which appeared to be a glucoside. From the few studies on the cytokinin content of leaves, there is insufficient information to establish a relationship, based on cytokinins, between buds and leaves, although the inhibitory influence of leaves on bud growth is readily observed in many species. Similarly, there is no information on the endogenous cytokinin content of buds during dormancy induction, and presently, there is some evidence only to suggest cytokinins have a role in the release from dormancy.

It is not clear how cytokinins can cause dormancy release. The possibility that cytokinins act by counteracting or causing the breakdown of inhibitors, such as ABA, has not been investigated in woody species. Similarly, their interrelationship with other endogenous growth regulators is not clear. However, Alvim, Hewett and Saunders (1976) did find an inverse relationship between cytokinin content and abscisic acid levels in xylem sap at times of shoot growth and bud dormancy in willow. Low cytokinin activity and high ABA levels were found at the time of winter dormancy, whereas cytokinin activity was higher and peaked at the times of floral and leaf bud burst. Alvim et al. (1976) suggested that high ABA levels in the presence of low cytokinin levels may be responsible for maintaining dormancy over winter, and that bud burst, even under SD photoperiods, could have been

induced in the presence of high levels of cytokinins at a time of decreasing ABA levels. However, before any role for cytokinins in dormancy regulation is suggested, positive identification and quantitative determinations of the cytokinins that are present in the species under study need to be made. The lack of definitive bioassays has prevented accurate quantification, but with newer techniques (Horgan, 1980) this should be possible. The various forms of cytokinins may be active in a bioassay, but this does not necessarily indicate similar activities in the intact plant. Therefore, questions on occurrence, synthesis, transport and metabolism of cytokinins and on their relationship to other growth regulators need to be answered before their role in bud dormancy is determined. Presently, good *prima facie* evidence suggests that they may have a role in the control of bud dormancy.

1.5 NATURE AND SCOPE OF THE INVESTIGATION

At the commencement of the present investigation (1976) the literature up to and including 1975 was strongly supportive of the inhibitor hypothesis of dormancy (Wareing, 1969) with abscisic acid having a central role. However, the observations of Lenton, Perry and Saunders (1972) that ABA does not increase in apices during the photoperiodic induction of dormancy, and of Hocking and Hillman (1975) that exogenous applications of ABA did not cause the induction of bud dormancy in actively growing seedlings, questioned the role of ABA in the regulation of bud dormancy as projected by the inhibitor hypothesis. In view of the controversy and confusion these observations raise, it was decided to reconfirm and extend the experiments of Lenton, Perry and Saunders (1972) and Hocking and Hillman (1975) with particular emphasis given to determining endogenous free and bound (alkali-hydrolysable) ABA activity, and to ensuring (1) that the growth conditions (day/night temperatures) during the experimental period were more comparable to those likely to be encountered in the natural environment and (2) that actively growing buds are exposed to adequate levels of exogenous ABA.

On the assumption that ABA is not involved in dormancy regulation, it was also decided to re-examine, with the particular intention of

reconfirming or otherwise, some of the early studies on inhibitor β on which the inhibitor hypothesis is based.

The present investigation, therefore, re-examines the inhibitor hypothesis and the inhibitor-promoter hormone balance concept of bud dormancy, with specific reference to inhibitor β and ABA, using field grown saplings, seedlings, isolated shoots, and aseptically cultured shoot tips and buds. Abscisic acid's role in the induction and maintenance of bud dormancy is to be re-examined, and the possible involvement of short chain fatty acids in dormancy is to be investigated. In addition, the possibility of using aseptically cultured shoot tips of woody species, not only as a system for studying the effects of exogenously supplied growth regulators on shoot and bud growth, but also as a bioassay for the detection of dormancy-inducing substances, is also to be examined. It is hoped to resolve some of the confusion and controversy surrounding the role of ABA in dormancy regulation.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT MATERIAL

2.1.1 Experimental

2.1.1.1 Seedlings

The alder species *Alnus viridis* (green alder) was used. The seedlings, which had been raised from seed sown in the spring in plots at the Forest Research Institute Nursery, Rangiora, were lifted from the soil in the summer and transplanted into 15 cm plastic pots containing soil, peat and sand (2:2:1 :: v:v:v). The potted seedlings were placed on a 2 cm layer of moist gravel, contained in trays, and kept well watered whilst maintained under favourable growth conditions in growth rooms until required.

2.1.1.2 Mature trees

Several species were used in different studies.

(i) *Alnus viridis*. Shoots were collected from 1.5 m tall shrubs growing at the Rangiora nursery.

(ii) *Alnus glutinosa*. Leaves and apices were harvested from 7 m tall trees growing on a river bank at the University of Canterbury.

(iii) *Populus* species. Shoots were collected from mature trees of *Populus nigra* which had been topped to a height of 3.5 m the previous autumn. The topped trees formed a hedge at the University of Canterbury.

The following species were used for tissue culture studies: *Populus flevo, P. nigra, P. yunnanensis* and *P. tremoides*. Buds were collected from three year old seedlings growing at the Ministry of Works Nursery, Christchurch.

(iv) *Salix alba/babylonica*. Shoots were collected from a mature tree growing on the river bank at the University of Canterbury.

2.1.1.3 Aseptically cultured shoots

For experimental purposes, adventitiously formed shoots of *Populus yunnanensis* were used. The apical portion was excised from shoots which had been maintained on medium 2 for four weeks.

Carbohydrate analysis was carried out on freeze dried tissue which had been kept in screw capped jars at room temperature.

2.1.2 Bioassay

2.1.2.2 Wheat (*Triticum aestivum* L.)

Untreated seeds of the cultivar Hilgendorf were obtained from a commercial source (Dalgety NZ Ltd). Seeds were stored at room temperature, in the dark.

2.1.2.2 Lettuce (*Latuca sativa*)

Seeds of the cultivar "Great Lakes" were obtained from a commercial source (Yates and Co.). The seeds were stored in foil containers, in a freezer, at -10^{o}C.

2.2 HARVEST AND COLLECTION PROCEDURES FOR PLANT MATERIAL

2.2.1 Leaves

The youngest, fully-expanded leaves were harvested, cut into thin transverse sections using scissors, and immediately placed into ice cold 80% MeOH (v/v in water) for extraction. Only those leaves within hand reach were harvested from similar mature trees, and most of the leaves, whether from mature trees or seedlings, were from nodes 3, 4 or 5 below the shoot apex. Harvesting was carried out between 1400 h and 1600 h for outside material, and 5 or 6 h after the start of the high light intensity photoperiod in the case of the controlled environment studies.

2.2.2 Apices

Apices were harvested as for leaves. Only apical buds from main shoots were harvested from mature trees, but all apices, including those from lateral shoots, were harvested from seedlings used in controlled

environment studies. When apical buds were still growing, the apices plus that portion of the apical shoot with leaves adjudged to be one-eighth expanded, were harvested for extraction and termed the apical extract. For apparently dormant buds, the apical meristems plus bud scales were harvested and extracted.

2.2.3 Shoots

Lengths of young shoots were cut and immediately placed in buckets containing distilled water. Collection was limited to those shoots within hand reach, and was carried out between 1400-1600 h. The shoots were recut in the laboratory, such that each shoot consisted of 10 apparently well formed dormant buds (and leaves in some harvests). The buds were labelled basipetally by numbering. The recut shoot bases were immediately placed in the treatment solutions (50 ml) contained in 100 ml beakers. In some experiments, the leaves of half the shoots, or half (5) of the leaves from each shoot, were removed before recutting and immersion of shoot bases in treatment solutions.

2.2.4 Aseptically Cultured Shoots

Shoot tips (5 to 8 mm long) were excised from 20-60 mm long mother shoots (grown on M2). The excised shoot trips were transferred on to the treatment media. All experimental shoots used for a single experiment were harvested from mother shoots of the same age (4 to 6 weeks).

For the excision of shoot tips from mother shoots, the shoot plus some agar was removed from the culture tube and placed on a 9 cm petri dish base. The tissue was left embedded in the agar, whilst the shoot tips were excised to avoid any wilting of tissue. A drop of sterile water was used to submerge that portion of the shoot where the cut was to be made. Alternatively, when tissue became separated from the agar during removal from the culture tubes, a pool of sterile water was formed on the surface of the petri dish base and the tissue, especially the shoot bases, submerged within this pool.

Shoots from the various treatments were harvested from the culture tubes and gently squeezed between two layers of absorbent-paper tissue before fresh weights and lengths were determined. In some cases it was necessary to rinse the shoots in distilled water to wash off any

residual agar, before wiping with paper tissue. For sugar and starch determinations, the harvested shoots were thoroughly rinsed in three, 3 min washes of distilled water before freeze drying.

2.3 EXTRACTION, FRACTIONATION AND ASSAY OF UNKNOWNS

2.3.1 Inhibitor β

2.3.1.1 Extraction

Plant tissue was extracted in 80% methanol (MeOH) (v/v in water) at 4°C for 72 hours, or until all the chlorophyll was extracted. The tissue had been placed in one litre conical flasks containing a solvent volume to tissue fresh weight ratio of 50:1 (v:w), and stirred occasionally. The flasks were kept in the dark. The solvent was decanted after 24 hours, and replaced by one half the original volume of solvent. This procedure was repeated every 12 hours, and the extracts bulked. The dry weights of tissue were then determined. Any residue in the bulked extract was removed by filtration through a Buchner funnel using 9 cm Whatman GP filter paper.

The filtered extract was reduced to an aqueous solution by evaporating the MeOH under vacuum at 35°C, using a rotary evaporator and water bath. The pH of the aqueous extract was adjusted to 2.5 - 3.0 with conc. H_2SO_4, and partitioned four times with one third its volume of diethyl ether in a 100 ml separatory funnel. The separatory funnel was gently shaken or swirled for 2 min, and the layers allowed to partition for at least 3 min. The ether phases, which contained the acidic ether-soluble substances, were bulked and the aqueous phase discarded. The ether phase was then partitioned four times against one quarter of its volume of 5% sodium bicarbonate ($NaHCO_3$, pH = 9) and discarded. The bicarbonate phases were bulked and after adjusting the pH to 2.5 to 3.0 with conc. H_2SO_4, the solution was repartitioned four times with one third its volume of diethyl ether. This acid, ether-soluble fraction was then reduced to dryness, sealed in a small test tube using solvent washed silver foil, and stored at -15°C until required for paper chromatography.

2.3.1.2 Paper chromatography

The dried extract, containing ether-soluble components, was dissolved in a small volume (3-15 drops) of a 1:1 (v:v) mixture of methanol and ether and an aliquot, usually equivalent to 0.25 g dry weight of leaf material or 0.20 g dry weight of apical material, streaked on to a 20 × 20 cm Whatman No. 1 chromatography sheet (Pattern S). All the chromatography sheets used in the study were from the same batch labelled "Control no. 81901", and were washed three times in 80% MeOH (v:v in water) for 3 hours before use. The aliquot of extract was loaded on to the paper by using a pasteur pipette, which had been modified over a hot flame so that a very fine capillary tube was formed.

After loading, the chromatographs were developed by ascending chromatography in isopropanol : ammonia : water (10:1:1 :: v:v:v) solvent. A stainless steel frame, which held four chromatographs, was used to support the paper during the development time of approximately 4.5 to 5.0 hours. In each set of four chromatographs, that could be inserted into the steel frame, three were loaded with extract and the fourth only with a few drops of the MeOH-ether solvent. This chromatograph served as the solvent control. The solvent tank, which was lined with chromatography paper, was sealed and maintained at room temperature, in darkness, during development. The solvent was allowed to run to a pencil line 15 cm from the application zone, which was 2.5 cm from the base of the chromatography sheet. The developing solvent was replaced every 2 days, or after 20 chromatographs had been developed.

Following development, the chromatographs were air dried in darkness for approx. 12 hours at room temperature, and then divided into 15 equal R_F sections. Each R_F section was assayed using wheat coleoptiles or lettuce hypocotyls. When chromatographs were not bioassayed within 12 to 24 hours, they were stored in plastic containers at $-15^{o}C$. Solvent washed silver foil was placed between each chromatograph during storage, and most were assayed within 72 hours of development.

In addition to the chromatography of extracts, a series of ABA standards were also chromatographed. Aliquots (2 ml) of $10^{-4}M$, $10^{-5}M$, $10^{-6}M$, $10^{-7}M$, $10^{-8}M$ and $10^{-9}M$ ABA were taken to dryness in vacuo, at $30^{o}C$. These standards were then chromatographed as described above for extracts. The standards represented a final ABA concentration of

51.2 µg, 5.12 µg, 0.512 µg, 0.0512 µg, 0.00512 µg and 0.000512 µg, respectively. Three replicates of each standard were chromatographed.

2.3.1.3 Bioassays

Paper chromatographs of tissue extracts, synthetic ABA and MeOH:ether (1:1::v:v) (solvent-run control) were divied into 15 equal R_F sections and each section placed into a 5.1 cm × 1.8 cm glass petri dish containing 2 ml of distilled H_2O, if assayed on wheat coleoptiles or 1.5 ml if assayed on lettuce hypocotyls. Each R_F section was soaked in the aqueous solution for at least 1 h before bioassay. Unless otherwise stated, the inhibitor β bioassays were performed on triplicate aliquots taken from a minimum of three extracts at each harvest. Hence, a minimum of nine chromatographs were assayed for each harvest date using one bioassay.

The response of wheat coleoptiles and lettuce hypocotyls to the presence of ABA in solution was also tested at regular intervals during the entire analysis. For each series of bioassays, that corresponded to the beginning and end of each separate harvest date, a series of ABA standards (10^{-4}M to 10^{-8}M) were tested. This was to check whether the plant material used in the bioassays remained stable throughout. A random selection of typical response curves in figures 9 and 14 confirmed that there were no major changes in the shape and slope of the curves. On average, 12 bioassays were completed per week.

(a) Wheat Coleoptile Assay. The method used was based on that described by Bentley and Housley (1954). Wheat seeds, soaked for 1 h in aerated water at room temperature, in the light, were germinated in darkness on moist filter paper at 24°C for 72 hours. The filter paper was placed on a 32.5 × 25 cm perforated stainless steel plate which was inserted into a 36 × 28.5 × 6.5 cm stainless steel growth tray such that the plate rested horizontally 3 cm above the base of the tray. The tray was filled with distilled water to a level immediately below the plate. The edges of the filter paper were submerged in the water. During germination, a loosely fitting metal cover was placed over the tray.

With illumination provided by a green safe light, etiolated coleoptiles between 25 mm and 35 mm in length were selected, placed on a guillotine, and 10 mm sections severed 3 mm below the tip.

The guillotine with two razors could sever 10 coleoptiles simultaneously, and these were immediately placed into a petri dish containing the R_F section and water. Three petri dishes containing only water were also assayed to serve as water controls. Enough coleoptile sections to bioassay one chromatograph and the water controls, could be obtained from the seeds germinated on one growth tray. All petri dishes were placed into 26.5 × 19.5 × 9.0 cm plastic boxes lined previously with moist paper and the lid loosely replaced. After 24 hours at 24°C in the dark, the lengths of the coleoptile sections were measured to the nearest millimetre using a shadow technique. A photographic enlarger set at a magnification of 2.5 times was used. Each chromatograph with its 15 R_F sections and water controls took approx. 45 min to set up and 40 min to measure.

The mean length of the 10 coleoptile sections in each dish was expressed as a percentage of the water controls. Replicate (R_F) means were then averaged and this mean ± S.E. (standard error) was plotted against the position of the R_F section on the chromatograph to give histograms.

The response of wheat coleoptile sections to ABA solutions was tested by adding 2 ml of the solution to a triplicate series of petri dishes and incubating as above. The following concentrations were employed: 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M. A dose-response curve was constructed.

(b) Lettuce hypocotyl assay. The bioassay procedure was modified from that described by Goto (1978). Seeds were germinated in darkness on moist 15 cm filter paper circles that had been placed in plastic petri dishes at 24°C for 24 hours. From each of the plastic petri dishes, a sufficient number of seedlings was obtained to bioassay one chromatograph and the water controls. Under dim light, uniform seedlings with radicles approx. 2 mm long were selected and 16 of these seedlings were placed in each of the petri dishes containing one chromatograph section soaked in 1.5 ml of water. A triplicate series of petri dishes, each with filter paper circles (4 cm Whatman No. 1) and 1.5 ml of water, was used as the water controls. All petri dishes were then placed in 26.5 × 19.5 × 9.0 cm plastic boxes that had been lined previously with moist filter paper and the lids loosely replaced. The boxes were transferred to a dark room and the bioassay incubated at

24°C for 72 hours. After three days, the lengths of the hypocotyls were measured to the nearest millimetre using a shadow technique, which involved a photographic enlarger set at a magnification of 2.5. Each chromatograph took 50 min to set up and 50 min to measure.

The mean of 16 hypocotyls in each dish was calculated and expressed as a percentage of the water control. The means (± S.E.'s) of the replicate chromatograph sections were then plotted against the position of the section on the chromatograph, to give histograms.

The response of lettuce hypocotyls to the presence of ABA in the incubation medium was tested by adding 1.5 ml of an ABA solution to a triplicate series of petri dishes, each containing one filter paper circle, and incubating as described abvoe. The following concentrations were used: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. A standard curve hypocotyl length versus ABA concentration was constructed.

2.3.2 Abscisic Acid

2.3.2.1 Extraction

Free and bound (alkali-hydrolysable) abscisic acid was extracted from the same tissue. The extraction and purification procedures were based on the method of Lenton, Perry and Saunders (1971) and are outlined in figure 1.

(a) Free ABA. Plant tissue was extracted in 80% (v/v) aqueous MeOH for 72 hours at 4°C, with occasional stirring. The solvent was added to the tissue in one litre conical flasks until a solvent volume to tissue fresh weight ratio of 50:1 (ml:g) had been established. The solvent was decanted from the tissue after 24 hours and then every 12 hours, and replaced with one half the original volume each time. The five extracts were bulked and any insoluble residue was removed by filtering through a Buchner funnel using 9.0 cm Whatman GP filter paper. The residue and extracted tissue were transferred to a drying oven for dry weight determinations.

Following the filtration step, the methanolic extract was reduced to an aqueous solution on a rotary evaporator at 35°C *in vacuo*. The aqueous solution was adjusted to pH 3 with conc. HCl and extracted three times with one third its own volume of ethyl acetate (EtoAc).

Solvent partitioning was achieved by using a 100 ml separatory funnel. The EtoAc phases were bulked and the aqueous phase retained for extraction of the alkali-hydrolysable or bound ABA. The EtoAc extract was partitioned three times against one third its own volume of 5% $NaHCO_3$ solution. The combined bicarbonate phase was adjusted to pH 3 with conc. H_2SO_4 and partitioned three times with half its volume of diethyl ether. At all times, phase separation during solvent partitioning was allowed to occur for at least 5 min after the separatory funnel had been shaken gently and swirled for 2 min.

The ether phase was reduced to dryness *in vacuo* at room temperature using a rotary evaporator. The dry residue was redissolved in 50 ml of 5% $NaHCO_3$ solution and transferred to 150 ml beakers. The bicarbonate solution was slurried continuously for 1 hour with insoluble PVP (polyvinylpyrrolidone, Polyclar. AT). The PVP had been washed previously and the fines were removed by several cycles of suspension in distilled water. PVP in a dry powder form was added to the bicarbonate phase until no further colour change was observed in the added PVP. After 1 hour of PVP treatment, the mixture was filtered through a Buchner funnel using 5 cm Whatman No. 1 filter paper. The PVP was washed three times with 5 ml of 5% NaHCO and the combined bicarbonate solution was readjusted to pH 3 and extracted three times with one half its own volume of ether. The ether phase was reduced to dryness *in vacuo* at room temperature, and stored in a test tube at $-15^{o}C$ until thin layer chromatography.

(b) Bound ABA. The aqueous phase remaining after the first extraction with EtoAc was adjusted to pH 11 with conc. NaOH and kept at $60^{o}C$ for 1 hour. The solution was stirred occasionally and the temperature was maintained at $60^{o}C$ using a heated water bath. After cooling to room temperature, the solution was adjusted to pH 3 with conc. H_2SO_4. The solutions were then fractionated by solvent partitioning and slurried with PVP, as for free ABA. The final ether fraction was taken to dryness *in vacuo* at $35^{o}C$ and the dried extract was termed the alkali-hydrolysable ABA fraction, or the bound ABA fraction.

2.3.2.2 Thin layer chromatography

The dried extracts were redissolved in a small volume (approx. 15 drops) of a 1:1 (v:v) solution of MeOH:ether and line-loaded on to

20 × 20 cm aluminium backed plates of 250 μm thick silica gel GF_{254} (Riedel-de Haen), which had been eluted previously with ethanol : acetic acid (98:2 :: v:v) and air dried. Pasteur pipettes, modified as described in section 2.3.1.2, were used to line-load the extracts in 15 cm bands approx. 2.5 cm from the base of the chromatographs. Marker spots of authentic ABA were spotted at both ends of the loaded extract and were separated from the extract by a groove in the silica gel, 2.5 cm from and parallel to the two outer sides of the chromatograph.

The loaded chromatographs, in sets of eight, were developed in the solvent system, chloroform : MeOH : H_2O (75:22:3 :: v:v:v), by ascending chromatography until the solvent reached a groove 15 cm from the application band. The inside of the glass tank was lined with chromatography paper, and the tank was maintained at room temperature, in darkness, during the development time of approx. 120 min. Following development, the chromatographs were air dried in darkness for 10 min at room temperature, and the zones corresponding to the marker spots, when viewed under short wave U.V. radiation, were scraped off using a flat-ended glass rod. Generally, the zone removed was located at R_F 0.7 - 0.8. The silica gel removed from the chromatograph was washed with 3 ml of acetone : MeOH (1:1 :: v:v) in glass centrifuge tubes. The tubes were stirred vigorously on a vortex mixer for 1 min, and then centrifuged for 10 min at 30,000 rpm. The acetone : MeOH solvent was decanted into a second series of centrifuge tubes and the elution of the silica gel repeated twice. After elution the acetone : MeOH solution was dried *in vacuo* at 30°C using a test tube evaporator. The dried extract was then taken up in three drops of MeOH, in preparation for methylation.

2.3.2.3 Gas chromatography

Prior to chromatography, each extract was methylated with diazomethane using the small scale technique of Schlenk and Gellerman (1960). Two ethereal traps, connected in sequence, were used to trap the diazomethane. Briefly, diazomethane, dissolved in ice-cold ether, was added to the extract until the yellow colour of the ethereal solution persisted and the generation of bubbles ceased. The reaction was considered to be complete although all extracts were allowed to remain in the diazomethane ethereal solution for 10 min. After

methylation, the extracts were dried *in vacuo* at 30°C using a test tube evaporator and stored at -15°C before gas chromatography.

The methylated extracts were taken up in a known quantity, usually 1000 µℓ, of ice-cold pet. ether (40°-60°C, grade) and a microquantity, usually 0.5 µℓ or 1 µℓ, of the solution injected into a Tracor (Model 550) gas chromatograph fitted with a ^{63}Ni electron capture detector and linearizer. A 10 µℓ SEG microsyringe was used and the solvent flushing technique employed for all injections (Anon., 1977). Between each injection, the syringe was rinsed 10 times in each of three separate containers of pet. ether by fully loading and emptying the syringe.

Each sample was chromatographed at 195°C on 3% OV 17 coated on Chromosorb WHP 80/100 in 2 m × 2 mm glass columns. Oxygen free nitrogen at a flow rate of 50 ml min^{-1} was used as the carrier, while the same gas at a flow rate of 65 ml min^{-1} was used as a detector purge. The detector temperature was 295°C and the inlet and outlet port temperatures 250°C and 265°C, respectively. The relative pulse width setting was 0.25 and the standing current was at 2.5% of full scale deflection. One injection was made every 20 to 30 minutes depending on the base line response.

In addition to gas chromatography on OV 17, one replicate of each treatment was chromatographed on 3% OV 210, 3% OV 1, and 10% SE 30. All phases were coated on Chromosorb WHP 80/100, and the first two were packed in a 2 m × 2 mm glass column while the third was in a 2 m × 2 mm metal column. The detector temperature, inlet temperature and outlet temperature were the same as for the OV 17 column while the oven temperatures were 205°C, 205°C and 210°C for the OV 210, OV 1 and SE 30 columns, respectively. The relative pulse width setting and standing current were the same as for OV 17. The purge gas flow rate was increased to 80 ml min^{-1} for SE 30, and to greater than 100 ml min^{-1} for the OV 1 and OV 210 columns. Before use, all columns were conditioned overnight at temperatures 10°C greater than the final operating temperature.

The detector response, which was linear over the range 10 pg to 1000 pg of methyl-ABA (Me-ABA), was recorded on a Linear pen recorder. The peak heights of authentic Me-ABA standards were measured and a standard curve constructed twice each day. The abscisic acid in unknown

samples was determined from these curves.

For quantification purposes, the following sequence of injections on to columns was followed: standard (100 pg authentic Me-ABA), test sample, co-injection standard plus sample, standard, sample, standard. The relative peak heights were compared for agreement.

The identity of the presumed Me-ABA peak was confirmed by co-chromatography with authentic Me-ABA and by comparing retention times with authentic Me-ABA on each of the four columns. Confirmation by isomerization under U.V. light (Lenton et al., 1971) was also attempted and the mass spectra of the suspect ABA peak obtained for one leaf and one apical sample.

Determination of extraction efficiency or losses during extraction and purification was carried out using mixed isomers of synthetic ± ABA. A known amount (500 ng) was added to an aqueous solution, and the synthetic ABA extracted and purified in the same manner as the tissue extracts. The recovery (or extraction efficiency) was 60% and all values reported, are values after correction for losses.

2.3.2.4 U.V. spectroscopy

A Pye Unicam Ultraviolet Spectrophotometer was used to obtain U.V. spectrum of autoclaved and non autoclaved ABA solution. A scan speed of 2 nm sec^{-1} over the absorbance range 190 nm to 330 nm was used with chart speed of 10 sec cm^{-1}. The U.V. spectra of an aliquot (10 ml) of aqueous ABA (10^{-4}M) and ABA which had been added to a solution containing inorganic salts and organic supplements (see section 2.7) was obtained before and after autoclaving. The ABA was extracted from the aqueous solution at pH 2.5 with EtoAc as described in section 2.3.2.1. The dried extract was taken up in spectroscopic grade EtoAc. A qualitative comparison between autoclaved and non autoclaved ABA was made.

2.3.2.5 Gas chromatography - mass spectrometry

For confirmation of the ABA peak found on gas chromatograph profiles, two leaf extracts and one apical extract were further analysed by GC-MS. This work was carried out by Dr John Shaw, Applied Biochemistry Division, D.S.I.R. Mass spectra were obtained using an A.E.I. MS30 double beam mass spectrometer fitted with an all-glass

molecular jet separator kept at 250°C, operating at an ionization voltage of 70 eV with a scanning rate of 10 sec decade^{-1}. A 3% OV 17 column, similar to that used previously, was operated at 180°C with helium as carrier gas, at a flow rate of 40 ml min^{-1}.

2.3.3 Carbohydrates

The freeze dried plant material was ground, using a glass rod, to pass through a 2 mm screen.

2.3.3.1 Total soluble sugars

The method was based on that of Haslemore and Roughan (1976). Dried plant material (<100 mg) was extracted with 10 ml of 62.5% (v/v) aqueous MeOH for 15 min at 55°C, using screw capped borosilicate glass culture tubes (16 × 125 mm) with Teflon-faced caps. The temperature was maintained at 55°C by the use of a thermostatically controlled water bath. The samples were cooled to room temperature and centrifuged. Aliquots (4 ml) were transferred to a second set of capped culture tubes, each containing 0.1 ml of saturated aqueous solution of neutral lead acetate. (The original culture tubes containing plant material were retained for starch analysis, with the remaining volume of 62.5% MeOH being decanted prior to this.) After 10 min at room temperature and occasional shaking, 5 ml of chloroform was added, and the tubes capped and shaken vigorously. The tubes were centrifuged briefly for 1 min at 20,000 rpm to aid phase separation. Aliquots (50 µl) were then removed from the upper aqueous phases, and added to 1 ml of 5% phenol (w/v in water) and 4 ml of 98% sulphuric acid. The tubes were left to cool at room temperature before the absorbances were read.

A sucrose standard solution was made up at a concentration of 10 mg ml^{-1} in 62.5% MeOH. A series of sucrose standards were prepared from this by diluting 0, 0.5, 1.0 and 1.5 ml sucrose standard solution to 10 ml with 62.5% MeOH. The 10 ml of 62.5% MeOH was used as the blank control. Aliquots of 4 ml were removed from each of the prepared standards and treated with lead acetate, etc., in the same manner as the unknowns, to give standards equivalent to 0, 5, 10 and 15% soluble sugars on a dry weight basis respectively.

A WPA Co 65 Colorimeter fitted with filter no. 2 was used for absorbance readings, and each sample was determined by inserting each

culture tube, which had been wiped dry with paper tissue, directly into the light chamber. There was no significant difference between readings due to the use of different culture tubes (K. Sharrock, pers. comm.).

A standard curve was plotted from the standards, and sugar levels in the samples determined from this.

2.3.3.2 Starch

Extraction of starch was based on the method of McCready, Guggolz, Silviera and Owens (1950). Methanol (3 ml) was added to the residual plant material following soluble sugars extraction, and the culture tubes placed in boiling water (100°C) for 5 min. The procedure was repeated and the MeOH washings discarded after brief centrifuging (3 min, 20,000 rpm). The final washing was tested qualitatively with fresh anthrone reagent (2 g of anthrone dissolved in one litre of cold 95% sulphuric acid) and found to be negative. After the final centrifuging, 5 ml of distilled water and 3.25 ml of 52% perchloric acid were added to the sugar free plant residue. (A stock solution of 52% perchloric acid was made by adding 52 ml of a 70% perchloric acid solution to 18 ml of distilled water, and was stored at room temperature in glass stoppered, dark stained jars.) The aqueous perchloric acid solution was left at room temperature for 20 min, with the culture tubes being vigorously shaken every 5 min. The aqueous solution was poured into a 100 ml volumetric flask and the solubilization procedure using perchloric acid repeated. Following solubilization the combined solutions were diluted to 100 ml with H_2O and filtered through 9 cm Whatman No. 1 filter paper. The first 5 ml were discarded, and the remainder retained for starch estimation by the anthrone method. A 5 ml aliquot of the filtered starch solution was pipetted into a 16 × 125 mm screw capped borosilicate glass tube and 10 ml of fresh anthrone reagent (2 g of anthrone dissolved in one litre of cold 95% H_2SO_4) added. The anthrone was stored in a glass-stoppered, dark stained glass jar at 4°C. Each culture tube was shaken thoroughly and heated to 100°C for 7.5 min by placing in boiling water. The tubes were then transferred to a water bath at 25°C, for rapid cooling. After approx. 5 min, the colour intensities (absorbances) of the tubes containing samples were read using a WPA Co 65 Colorimeter fitted with filter no. 6. The culture tubes were wiped dry with soft paper tissue and inserted directly into the colorimeter for absorbance readings.

A stock solution of glucose was made by dissolving 100 mg of anhydrous glucose in 100 ml of water, and a series of glucose standards were prepared from this by adding 6.5 ml of perchloric acid to 0, 0.5, 1.0 and 2.0 ml aliquots of stock solution and diluting to 100 ml with distilled water. The standards were filtered and 5 ml of each subjected to anthrone treatment, etc. in the same manner as the unknowns.

A standard curve was constructed from the standard glucose determinations, and unknowns calculated from this. The glucose found was multiplied by 0.90 for conversion to starch units.

2.3.4 Phenolics

Three reagents, which were used to detect the presence of phenolics, were sprayed on to chromatographs and any colour reactions seen under visible and U.V. light noted. The reagents were 0.2% Ferric chloride (w/v in water), diazotised p-nitroaniline and diazotised sulphanilic acid. Diazotised p-nitroaniline was made by dissolving 0.3 g of p-nitroaniline in 99.7 ml of 8% HCl (v/v in water). An aliquot (25 ml) of the 0.3% p-nitroaniline solution was then mixed with 1.5 ml of 5% sodium nitrite (w/v in water) immediately before spraying. The application was followed by one of 20% sodium carbonate (w/v in water). The diazotised sulphanilic acid was prepared as for diazotised p-nitroaniline, but sulphanilic acid was used instead of p-nitroaniline.

2.3.5 Dry Weights

All dry weights (DW) of tissues were determined following solvent extraction, except in the case of the tissue used for sugar-starch determinations where dry weights were determined on freeze dried material prior to solvent extraction. After solvent extraction, tissue was dried for a minimum of 72 hours, in an oven at 110^{o}C. The dried tissue was weighed daily until consistent readings within ± 1% were obtained.

2.4 CONTROLLED ENVIRONMENT FACILITIES

2.4.1 Growth Rooms

Temperature controlled growth rooms situated in the Botany Department, University of Canterbury, were used. Temperature was maintained at 20°C ±1.5°C and lighting was provided by fifty 65W "colour 33" fluorescent tubes supplemented by twenty-four 15W tungsten filament bulbs. The photon flux density was 214 μE m^{-2} s^{-1}. A 16 hour photoperiod was employed for long day (LD) treatments. Humidity was maintained by placing trays of water in the room.

2.4.2 Growth Cabinet

A Sherer growth cabinet with photoperiod and day/night temperature programming was used. Temperature control was ±0.5°C and lighting was provided by 12 high output fluorescent tubes supplemented with six tungsten filament bulbs. The photon flux density was 230 μE m^{-2} s^{-1}. For long day (LD) treatments, a photoperiod of 16 hours was employed. The LD photoperiod consisted of 8 hours (0800 h to 1600 h) of high intensity illumination, supplied from the fluorescent tubes plus the tungsten bulbs, and 8 hours (1600 h to 2400 h) of low intensity light from the tungsten bulbs alone. The short day (SD) treatments consisted of 8 hours (0800 h to 1600 h) of high intensity illumination only and 16 hours of darkness. The day temperature in both LD and SD treatments was programmed for the period the fluorescent tubes were on. The remainder of the time was considered the night temperature. The day temperature was 14°C ±0.5°C and the night temperature 10°C ±0.5°C.

2.5 PREPARATION OF MEDIA

The composition of the basal medium used for aseptically cultured shoots was modified from Murashige and Skoog (1962) and was as described by Whitehead and Giles (1977) (Table 2.1). Stock solutions of inorganic nutrients (minus FeEDTA) and organic supplements (minus sucrose) were made up at 10 times the required concentration in one litre and 500 ml flasks, respectively. The inorganic nutrients stock solution was stored at 4°C in darkness, whereas 50 ml aliquots of the organic supplements stock solution were stored in screw capped glass jars at -15°C in

Table 2.1 Composition of medium used for studies with aseptically cultured shoots. Modified from Murashige and Skoog (1962) and Whitehead and Giles (1977). All media consisted of inorganic nutrients and organic supplements in quantities shown, except for ½ MS which had half the quantities shown. Weights in mg ℓ^{-1}.

(a) Inorganic nutrients

NH_4NO_3	1650	KNO_3	1900
$CaCl_2.2H_2O$	440	$MgSO_4 7H_2O$	370
KH_2PO_4	170	H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3	$ZnSO_4.4H_2O$	8.6
KI	0.83	$Na_2MoO_4 2H_2O$	0.25
$CuSO_4.5H_2O$	0.025	$CoCl_2.6H_2O$	0.025
FeEDTA	65.1	Agar	800

(b) Organic supplements

Nicotinic acid	0.5	Lysine	100
Pyridoxin-HCl	0.1	Sucrose	20 000
Thiamin-HCl	0.1	Adenine sulphate	20
Inositol	100		

(c) Growth substances

Medium	Benzyl adenine	Napthalene acetic acid
M0	0.5	0
M1	0.2	0
M2	0.1	0.02
M3	0.2	0.02
½ MS	0.2	0

darkness. Stock solutions of growth regulators were made up at the following concentrations: BA at 10 mg ℓ^{-1}, NAA at 4 mg per 100 ml. These were stored at $4^{o}C$, in darkness.

One litre of medium was prepared by dissolving 20 g of sucrose and 65.6 mg FeEDTA in 600 ml of distilled water, adding 100 ml of the inorganic salts stock solution, one jar (50 ml) of organic supplements, and 50 ml and 20 ml of BA solution for medium 0 and medium 1, respectively, or 10 ml BA plus 0.5 ml NAA for medium 2, or 20 ml BA

plus 0.5 ml NAA for medium 3. For the preparation of the media designated ½ MS (in Table 1), the inorganic salts and organic supplements were added at half strength and 20 ml of BA was added to make one litre. The dissolved solution of salts, organic supplements and growth regulators was made up to one litre with distilled water and the pH changed to 5.8 with 2N HCl. The media were solidified with agar (Difco Bacto); 8 mg of agar was added into each culture tube containing 10 ml of medium. The 10 ml aliquots of liquid medium were dispensed into 25 × 150 mm glass test tubes using a macrosyringe. A bung of cotton wool contained within muslin was inserted into the top of the test tube and an aluminium foil cap placed over the bung. The sides of the foil cap were pressed firmly against the outside of the test tube and the test tubes placed into metal racks for sterilization.

In addition to M0, M1 and M2, medium containing medium 2 plus various concentrations of ABA, GA_3, AMO 1618, C5 and C10, and crude extract, was also prepared. Aliquots of the growth regulator solutions were added before autoclaving, such that the initial concentration in the medium was 10 mg ℓ^{-1}, 1 mg ℓ^{-} , 0.1 mg ℓ^{-1} or 0.01 mg ℓ^{-1} (in the case of ABA, GA_3 and AMO 1618) and 100 mg ℓ^{-1}, 50 mg ℓ^{-1}, 10 mg ℓ^{-1} and 0.1 mg ℓ^{-1} (in the case of C5 and C10). Serial dilutions of the crude extract represented the equivalent of 250 mg, 25 mg, 2.5 mg and 0.25 mg dry weight of tissue per litre of medium.

For the assay of chromatographs of leaf and apical extracts, the R_F sections (0.1 units) were added to culture tubes containing M2 before autoclaving.

2.6 ASEPTIC TECHNIQUE AND CULTURE

The test tubes containing basal or treatment media were sterilised by autoclaving at 120°C for 15-20 min, at 103 kPa. All subsequent operations were carried out in a Laminar flow, sterile air cabinet.

The aseptically cultured adventitious buds and shoots were manipulated using sterile instruments; scalpels (No. 11 blade), forceps and 4 mm glass rods. Between each single manipulation with tissue, these instruments were sterilised by dipping in 90% ethanol (v/v in water) and flaming.

The excision and manipulation of tissue were performed on the surface of a 9 cm sterile glass petri dish. The base was wiped with cotton wool soaked in 90% EtoH, and flamed and cooled after each transfer and manipulation of plant material. Sterile water was obtained by autoclaving distilled water.

Bud explants were excised from non-sterile shoots, dipped in 90% EtoH, flamed and incubated in 0.5% NaOCl (Janola solution) for 15 min. The explants were then rinsed in three 5 min washes of sterile water, and the outer bud scales removed using forceps and a scalpel. The isolated meristem was immediately placed on to medium 1 using a glass rod. The cultures were incubated in a growth room set at a 16 h photoperiod, at 25^{o}C. After 4-6 weeks of incubation on M1, adventitious bud formation and proliferation had occurred in those explants that were free of contamination. Apparently single, adventitious buds (or groups of buds for the LD/SD experiment) were isolated from the mass and counted. The isolated buds were transferred to medium 1 or medium 2 and incubated for 4 weeks. On those buds transferred back on to medium 1, further adventitious bud formation occurred and this recycling regeneration procedure was used to maintain a stock source of adventitious buds.

Multiple shoot development occurred on the apparently single, isolated buds incubated on medium 2 and these shoots, 20-60 mm long, were referred to as mother shoots. A stock of mother shoots, from which the experimental or treatment shoot tips were obtained, was maintained. The excised shoot tips (0.5 to 0.8 cm long) were transferred back on to M2 and incubated for 1 week, before transfer on to the treatment media. Only those shoot tips showing the least amount of callus or adventitious bud formation were selected and transferred for use in the experiments.

2.7 PREPARATION OF GROWTH REGULATOR SOLUTIONS

All growth regulators were made up at the highest concentration required, and all other concentrations were obtained by serial dilution of the respective stock solution using pipettes and volumetric flasks. All aqueous solutions were stored at 4^{o}C, in darkness, and where solutions were required for spray or "paint" treatments, 3 drops of

Tween 20 [polyoxyethylene sorbitan monolaurate (Sigma Chemical Co.)] were added to each litre of solution. All solutions were used within 8 weeks of preparation.

Typically, 52.8 mg of mixed isomers of synthetic ABA (Sigma Chemical Co.) was dissolved in a minimum amount of MeOH (approx. 10 to 100 µℓ) and this was added in drops to 1 litre of vigorously stirred water, at 30°C, to make a 2×10^{-4} M solution.

Likewise, 709.8 mg of 4 hydroxy-5-isopropyl-2-methylphenyl-trimethylammonium chloride piperidine carboxylate (AMO 1618) and 632 mg of 2-chloroethyltrimethyl ammonium chloride (CCC, BDH Chemicals) were prepared as for ABA, to give 2×10^{-3}M solutions.

The combined solution of ABA plus CCC, or ABA plus AMO 1618, was made by mixing 1:1 volumes of the respective stock solutions.

A 10^{-3}M aqueous solution of n-valeric or pentanoic acid (C5, Sigma Chemical Co.) was prepared by dissolving 102.1 mg in 1 litre of vigorously stirred water, at room temperature.

Caprylic or octanoic acid (C8, Sigma Chemical Co.) solutions were prepared by dissolving 144.2 mg in approx. 1 litre of boiling and the solution readjusted with distilled water, to 1 litre after cooling to room temperature.

Pelargonic or nonanoic acid (C9, Sigma Chemical Co.) and decanoic or capric acid (C10, Sigma Chemical Co.) were dissolved in a minimum amount of EtoH before adding to boiling water. However, both acids were only sparingly soluble in water at room temperature and did not remain in solution for more than 24 hours. Instead, nonanoic acid (15.82 mg) and capric acid (172.3 mg) were first dissolved in a minimal amount of 2 M KOH and then added dropwise to 1 litre of water at 80°C to make the respective 10^{-3}M solutions. The pH of each solution was readjusted to 6.8 before use.

For tissue culture experiments, 10 mg of ABA, AMO 1618, gibberellic acid (GA_3, Grade III, 90% pure, Sigma Chemical Co.), and 100 mg of C5 and C10 were weighed out and each of the solutions prepared as described above for the more concentrated solutions, to make 100 ml of solution. The GA_3 solution was prepared in the same manner as the 2×10^{-4}M ABA solution. Aliquots; 20 ml, 2 ml, 0.2 ml and 0.02 ml in the case of ABA, AMO 1618 and GA_3 solutions, and

20 ml, 10 ml, 2 ml and 0.2 ml in the case of C5 and C10, were pipetted and diluted to 20 ml with distilled water, and these were added to 180 ml of basal medium, which in this case had been prepared as for 1 litre but diluted to 900 ml only. Therefore, the final desired strength of the basal medium was achieved by dilution with the 20 ml of growth regulator solution (treatments) or 20 ml of water (control). The final concentrations of growth regulators (besides BA and NAA) in the medium were 100 mg ℓ^{-1}, 1 mg ℓ^{-1}, 0.1 mg ℓ^{-1}, 0.01 mg ℓ^{-1} and 0 mg ℓ^{-1} in the case of ABA, AMO 1618 and GA_3, and 100 mg ℓ^{-1}, 50 mg ℓ^{-1}, 10 mg ℓ^{-1}, 1 mg ℓ^{-1} and 0 mg ℓ^{-1} in the case of the two fatty acids, C5 and C10.

Benzyl adenine (6-benzylaminopurine or BA, Sigma Chemical Co.) solution was prepared by dissolving 10 mg in minimal amounts of IN NaOH. This was added dropwise to 1 litre of vigorously stirred water. The pH was readjusted to 6.5.

A quantity, 4 mg, of napthaleneacetic acid (NAA, Sigma Chemical Co.) was dissolved in a minimal amount of MeOH (approx. 100 µℓ) and then added to 100 ml of vigorously stirred water to make a stock solution.

All growth regulator solutions used for the tissue culture studies were kept in a refrigerator at $4^{o}C$.

For abscisic acid standards used in gas chromatography, 5 mg of mixed isomers of synthetic ±ABA (Sigma Chemical Co.) were dissolved in 1 litre of twice distilled MeOH. An aliquot, 1 ml, was pipetted and diluted to 10 ml with MeOH to give an ABA standard of 500 pg $\mu\ell^{-1}$. Other standards were prepared by serial dilution to give the following standards; 250, 125, 100, 75 and 50 pg $\mu\ell^{-1}$. The standards made up in MeOH were taken to dryness, methylated and redissolved in the required amount of twice distilled pet. ether.

The phaseic acid standard was prepared from a stock solution in MeOH (a gift from B. Loveys, CSIRO, Australia). A quantity, 2 µg, was methylated and taken up in pet. ether to give a final concentration of 100 pg $\mu\ell^{-1}$.

2.8 CHEMICALS, REAGENTS AND GLASSWARE

Where possible, Analar grade chemicals and reagents were used.

All solvents were redistilled, once before general use and twice when used for chromatographic purposes.

Aqueous solutions were made up with distilled water.

All glassware was soaked after use in a solution of Pyroneg for 12 to 24 hours, washed repeatedly in running tap water, rinsed in distilled water and dried in an oven before re-use.

Glass-stoppered borosilicate glass containers were used wherever possible.

2.9 EXPERIMENTAL AND DESIGN

2.9.1 Studies on Photoinduction of Dormancy

2.9.1.1 Endogenous inhibitor β content

Changes in the inhibitor β content of leaves and apices during the photoinduction of dormancy (transfer of plants from LD photoperiods to SD photoperiods) were followed, using the wheat coleoptile and lettuce hypocotyl assays. The experiment was conducted in a growth cabinet set at LD and SD photoperiods with night and day temperatures of $10^{o}C$ and $14^{o}C$, respectively.

Alder viridis seedlings (20) were used in the study and the youngest, fully-expanded, mature leaves and apices were harvested separately from three sets of randomly paired plants on days 0, 7 and 15 of SD treatment. Of the two remaining plants, the mature leaves and apices were harvested from one plant on day 28 of SD treatment and the other plant was returned to LD's. The heights of the seedlings were measured at regular intervals.

For apical extracts, aliquots representing the equivalent of 0.2 g dry weight of apical tissue were bioassayed, whereas the equivalent of 0.25 g dry weight of leaf material was used for the assay of leaf extracts. For selected extracts, one from day 0 and one from day 15, serial dilutions equivalent to 0.025, 0.125 and 0.50 g dry weight of leaf material and 0.14 g dry weight of apical material (made up of combined

apical extracts) were also bioassayed.

Extracts (3) were prepared using the same solvents as those used during extraction and purification but in the absence of plant material. These extracts were termed blank extracts and served as controls.

The bioassay data was recorded as histograms with the mean of the replicate (3) chromatography sections (15) plotted against the R_F of that section.

2.9.1.2 Endogenous abscisic acid levels

The free and bound (presumed to be the glycosyl ester) abscisic acid content of leaves and apices following the photoinduction of dormancy (transfer of plants from LD to SD photoperiods) was determined by gas chromatography methods. A growth cabinet set at LD and SD photoperiods with $10^{o}C$ night and $14^{o}C$ day temperature was used for the experiment.

Leaves and apices were harvested on day 0 and 12 of SD treatment. At each harvest, three pairs of randomly selected plants were harvested giving three leaf extracts and three apical extracts for ABA determinations. On day 20 of SD treatment, the eight plants that remained after the first two harvests were transferred to growth rooms set at LD photoperiods at $25^{o}C$.

The amount of free and bound ABA in each extract was expressed as $\mu g\ kg^{-1}$ dry weight of tissue.

2.9.2 Studies on the Seasonal Variation in Inhibitor β

Changes in the inhibitor β content of leaves and apices during the autumn period were determined using the wheat coleoptile and lettuce hypocotyl bioassays.

Apices and leaves were harvested separately from similar mature common alder (*Alnus glutinosa*) trees during the autumn period, at approx. three weekly intervals from 5 March through to 15 May, 1977. The harvested dates were 7 March, 25 March, 15 April, 28 April and 15 May, designated H1, H2, H3, H4 and H5 respectively.

At each harvest, five leaf extracts, each of approx. 12 g fresh weight of leaf material, and three apical extracts, each of approx. 5 g

fresh weight, were made. Each extract was purified and aliquots prepared for bioassays on wheat and lettuce. The aliquot of leaf extract chromatographed was equivalent to 0.25 g dry weight of material whereas for each apical extract, the equivalent of 0.2 g dry weight of apical material was assayed.

The assay of inhibitor β was performed on triplicate aliquots taken from a minimum of three extracts at each harvest. In addition, for one H1 extract and one H5 extract, serial dilutions equivalent to 0.025, 0.125 and 0.375 g dry weight of leaf material in the case of leaf extract, and 0.02 g and 0.01 g dry weight of apical material in the case of apical extracts, were also bioassayed.

Appropriate controls, including solvent controls (three blank extracts as described in 2.9.1.1) were also prepared and assayed.

The results of the bioassay of the replicate (3) set of chromatographs of each replicate (3) extract, at each harvest date, were presented as histograms.

The experiment was repeated by the harvest of tissue during the autumn of 1978 and 1979. The harvest dates in 1978 were; 13 March, 14 April and 2 May, designated H1, H2 and H2, respectively, and in 1979; 5 March, 13 March and 4 April, designated H2, H2 and H3, respectively.

2.9.3 Studies on the Induction of Bud Dormancy

2.9.3.1 Root pruning

The influence of root pruning on the induction of bud dormancy under conditions favourable to growth was tested.

Potted, green alder seedlings were used in the study and the experiment was conducted in growth rooms under LD photoperiods at 20°C. The seedlings, with the soil intact, were removed from the pots and a series of cuts through the roots and soil were made with a pair of scissors, such that the proportion of soil that remained attached to the roots represented approx. one third of its original pot volume with the main axis of the undisturbed volume still centered on the main root. All roots, except those young or short roots located totally within the undisturbed soil, were considered to have been pruned. The root pruned plant and the soil were repotted. A similar set of plants,

which had been removed from the pots but did not have their roots pruned, were used as controls. Five or six plants were used for each treatment and the experiment was repeated three times. The plants used in the third repeat had their roots severely pruned such that approximately one-sixth of the original soil volume remained undisturbed.

The heights of the plants were measured at regular intervals and the mean increase in height was plotted against time.

2.9.3.2 Exogenously applied growth regulators

The ability of various growth regulator solutions to induce bud dormancy in alder seedlings under conditions favourable to growth was tested. The following growth regulator solutions were tested; 10^{-4}M ABA, 10^{-3}M CCC, 10^{-4}M ABA + 10^{-3}M CCC, 10^{-3}M C5, 10^{-3}M C9 and 10^{-3}M C10. An aqueous solution containing Tween 20 was used as the control solution, and this was applied to a similar set of plants to those used for the treatments.

The growth regulators were applied to the foliage by spraying the plants, and to the apices by painting the solution on to the bud surface using fine brushes. The plants were treated daily, to the point of run off, for a 4 to 6 week period and the plants were maintained in a growth cabinet or a growth room, set at LD photoperiods, throughout the experiment. A constant day/night temperature of 20^{o}C was used when the experiment was conducted in the growth room, whereas a 10^{o}C night and 14^{o}C day temperature were used when the growth cabinet was used. The experiment was repeated three times (once in the growth cabinet and twice in the growth room), although not all the treatments were included at all times. Three replicate plants (growth cabinet experiment) or 5 or 6 replicate plants (growth room experiments) were used for treatment. In the first two experiments, the youngest fully-expanded leaf was partly immersed in the test solution in addition to spraying the plant and painting the apices. However, this was discontinued in the last experiment. The plants were in a fully randomised arrangement within the growth cabinet or growth room.

The heights of the treated plants were measured at regular intervals and the results presented as the mean percentage increase in height plotted against time.

The ability of solutions of abscisic acid (10^{-4}M) to arrest growth and induce bud dormancy in excised growing shoots was also tested. Lengths of young growing shoots (20) with 10 apparently well developed buds and leaves were collected from green alder seedlings maintained in a growth room, set at LD photoperiods and 20°C. The apical bud on each shoot was actively growing at the time of collection. The cut bases were placed into a beaker of ABA solution and the leaves from half the shoots were removed. The excised shoots were returned to and incubated in the same growth room.

The number of growing buds at each node position (labelled basipetally) was recorded at weekly intervals.

2.9.4 Studies on the Maintenance of Bud Dormancy

The ability of ABA and other growth regulators to prolong the dormancy of buds after their transfer to an environment favouring growth was tested. Three species were used in the experiment.

2.9.4.1 *Populus nigra* "Italica"

Shoots with 10 apparently well formed dormant buds were harvested from mature trees on 24 July 1976 (designated late winter harvest). For each treatment, 10 shoots, each with 10 buds, which were labelled basipetally, had their cut bases placed into beakers containing the treatment solutions. Care was taken to avoid submerging the most basal bud in the solution, and each test solution was replicated four times. Each replicate of 10 shoots was incubated in one of the following test solutions: distilled H_2O, 10^{-4}M ABA, 10^{-5}M ABA, 10^{-3}M C5, 10^{-3}M C8, 10^{-3}M C9, 10^{-3}M CCC and 10^{-4}M ABA + 10^{-3}M CCC, in a growth room set at long day photoperiods at 20°C. The test solutions were refilled when the original volume (approx. 50ml) had fallen by half, and the solutions were renewed at weekly intervals.

The eight treatments were arranged within a growth room in a randomised complete block design with four blocks (replicates) and 10 shoots per treatment.

The number of buds which had grown visibly (swelling followed by shoot extension) was recorded at regular intervals for three weeks. A comparison was made between the mean percentage of growing buds (% bud burst) per treatment, and the number of growing buds at each

node was plotted against position on the stem, for each treatment.

2.9.4.2 *Alnus viridis*

Shoots were harvested and placed in treatment solutions as for poplar. In addition to the late winter harvest (21 July 1976), shoots were also harvested on 29 April 1977 (designated winter harvest) and 6 April 1979 (designated autumn harvest). For the first harvest date, the treatments consisted of the following test solutions: distilled water, 10^{-4}M ABA, 10^{-5}M ABA, 10^{-3}M CCC, 10^{-3}M C5, 10^{-3}M C8, 10^{-3}M C9 and 10^{-4}M ABA + 10^{-3}M CCC, whereas for the second and third harvest dates the following test solutions were used: distilled H_2O, 10^{-4}M ABA, 10^{-3}M CCC, 10^{-4}M ABA + 10^{-3}M CCC.

In the autumn and winter harvests, the leaves were still present on the shoot, and by removal of the leaves from half of the shoots this second factor (presence or absence of leaves) was tested against the following test solutions: distilled water, 10^{-4}M ABA, 10^{-3}M CCC and 10^{-4}M ABA + 10^{-3}M CCC. Each experimental unit comprised 10 shoots, with each treatment replicated four times in the case of the late winter and winter harvests and three times in the case of the autumn harvest.

All treatments were fully randomised, with a growth room set at the same conditions as for the incubation of the *Populus* shoots, i.e. LD's at 20^{o}C.

The number of buds that had grown at each position on the stem was recorded at regular intervals for 7 weeks (late winter and winter harvests) or 4 weeks (autumn harvest), and the mean percentage was plotted against the position on the stem. A comparison with the appropriate control was made between the mean percentage bud burst found for each treatment.

2.9.4.3 *Salix alba/babylonica*

In this species, the leaf factor (presence or absence) and the degree of influence of apical dominance on bud burst were investigated. The large number of treatments limited replication in this experiment, to two sets of 10 shoots for each treatment. In view of this, the experiment was repeated twice over two separate seasons.

Shoots with 10 apparently well developed dormant buds were harvested on 11 April 1978 and 16 April 1979. Both harvests were

designated autumn harvests.

Half the shoots with 10 foliated nodes, each with 10 apparently dormant buds, were partially or totally defoliated to determine the degree to which leaves control bud break. There were four defoliation treatments: no leaves removed, leaves removed from the first five apical nodes, leaves removed from the basal nodes, and all leaves removed. All the defoliation treatments were tested against the following test solutions: distilled water, 10^{-4}M ABA, 10^{-3}M CCC, 10^{-4}M ABA + 10^{-3}M CCC.

To determine whether apical dominance had any influence on bud break, the basal half of the shoot was isolated from the apical half. The leaf factor (absence or presence of leaves) was also included in these treatments. The leaves were removed from half of the divided shoots, and the shoots were incubated in the following test solutions: distilled H_2O, 10^{-4}M ABA, 10^{-3}M CCC, 10^{-4}M ABA + 10^{-3}M CCC.

All experiments were set up in a fully randomised arrangement in a growth room set at LD photoperiods, at 20^{o}C. After 4 weeks of incubation, the number of buds that had grown visibly was recorded. For each treatment, 10 shoots were used and each treatment was replicated twice each year. However, in 1978 several shoots (up to 50% of the total in some treatments) became diseased during incubation and only the results of the 1979 harvest are presented. Comparison of the mean percentage of growing buds for each treatment was made against the appropriate controls (distilled H_2O).

Shoots were harvested from a second, much younger tree of the same species, and bud break as influenced by the following test solutions: distilled water, 10^{-4}M ABA, 10^{-3}M CCC, 10^{-4}M ABA + 10^{-3}M CCC, and 10^{-3}M C10, was studied in the presence of leaves. The limited number of shoots harvested restricted the number of treatments and replicates. The experimental design was as for the mature tree; 10 shoots per treatment with each treatment replicated twice. The shoots were incubated in a growth room (LD's, 20^{o}C) for 4 weeks.

All the willow results were presented as a plot of the mean number of growing buds against position on the stem.

2.9.5 Studies with Aseptically Cultured Shoots

2.9.5.1 Suitability of species

The ability of bud explants of four poplar species (*Populus flevo, P. tremoides, P. nigra* "Italica" and *P. yunnanensis*), and one alder species (*Alnus viridis*), to produce adventitious buds and subsequently, 20 to 60 mm long shoots, was tested.

Dormant, and growing buds of alder, 100 of each species, were excised, sterilised and incubated on medium 1, under LD photoperiods in a growth room set at 25°C. For alder, 100 explants of growing and dormant buds were also incubated on each of the following media: M0, M3 and ½ MS. After 2 weeks, those buds that were free of contamination were transferred on to fresh medium 1, or in the case of alder, the fresh medium corresponding to the original medium. After 4 weeks, the number of adventitious buds that were visible was counted for 10 explants from each of the test species.

For all poplar species, 100 apparently single adventitious buds were excised and placed on medium 2. After 4 weeks of incubation, the number of test tubes in which the buds had developed into shoots greater than 20 mm was counted. In the case of green alder, where no adventitious buds had formed, 100 bud explants were transferred on to medium 2 and 100 explants on to M2 containing 1 mg ℓ^{-1} GA_3. The number of adventitious buds and shoots that developed, which exceeded 20 mm in length, was recorded.

2.9.5.2 Response to photoperiod

The effect of SD photoperiod on the growth of aseptically cultured shoots was tested.

Adventitious buds were excised in groups of two to four, from tissue grown on M1, and 200 of these multiple buds were transferred into culture tubes containing M2. After 3 weeks of incubation, under LD photoperiods, half (180) of the culture tubes were transferred to SD photoperiods. The fresh weights, lengths and dry weights of the shoots which developed were determined on days 0, 10 and 21 of SD treatment for both LD and SD treated shoots. Because of the need for aseptic culture, a destructive harvest technique was used; three replicates of 10 culture tubes were used for the harvest of shoots

at each harvest day. The culture tubes were randomly selected.

Any morphological changes that were visible in the shoots were recorded.

2.9.5.3 Assay for dormancy-inducing substances

(a) The possibility that dormancy-inducing substances may be present in the acidic ether-soluble fraction of dormant tissue was tested by the assay of chromatographs of extracts on aseptically cultured shoots.

The extracts assayed were two leaf extracts; equivalent to harvest 3 (H3) 1979 (see section 2.9.2) and harvest 2 (H2) (see section 2.9.1.1), and one apical extract equivalent to harvest 3 (H3) 1979 (see section 2.9.2). Aliquots equivalent to 0.25, 0.5 and 1.0 g dry weight of tissue were chromatographed and assayed in triplicate. Chromatographs were divided into 10 equal R_F sections after solvent development, and each R_F section was placed into the test tube containing medium 2, before autoclaving. Shoot tips (5 to 8 mm long) were excised from mother shoots, and after 1 week on M2, a single shoot was transferred into each culture tube containing M2 and the R_F section.

After 4 weeks of incubation (LD's, 25°C), the fresh weights and lengths of the shoots were determined, and from these the mean fresh weight and mean length for the triplicate R_F sections were plotted against the position of the R_F section on the chromatograph, to give histograms.

Any visible indications of dormancy were also noted.

(b) The possibility that dormancy-inducing substances may be present in the aqueous fraction of an 80% MeOH extract of dormant tissue was tested. The aqueous fraction was termed "crude extract", and the 80% MeOH extract was made of the aseptically cultured shoot material harvested after 21 days of SD treatment.

Aliquots of crude extract, equivalent to 25, 2.5, 0.25 and 0.025 mg dry weight of tissue were added to a series of culture tubes containing M2. The crude extract was added prior to autoclaving. A single shoot tip (5 to 8 mm long) was added to each culture tube, and after 4 weeks of incubation in a growth room set at LD's at 25°C, the growth response (fresh weight and length) was determined and

compared with the growth response on M2 alone. For each serial dilution of crude extract, 20 culture tubes (replicates) were set up.

Any visible indications of dormancy were noted.

2.9.5.4 The effect of growth regulating substances

The influence of various growth regulators on shoot and bud growth of aseptically cultured shoots was tested.

Shoot tips (5 to 8 mm long) were harvested from mother shoots and placed on to M2 for 1 week. Apparently single developing shoots were then selected and transferred to M2 containing one of the following concentrations of growth regulators: ABA at 10 mg ℓ^{-1}, 1 mg ℓ^{-1}, 0.1 mg ℓ^{-1} and 0.01 mg ℓ^{-1}, AMO 1618 at 10 mg ℓ^{-1}, 1 mg ℓ^{-1}, 0.1 mg ℓ^{-1} and 0.01 mg ℓ^{-1}, GA_3 at 10 mg ℓ^{-1}, 1 mg ℓ^{-1}, 0.1 mg ℓ^{-1} and 0.01 mg ℓ^{-1}, C5 at 100 mg ℓ^{-1}, 50 mg ℓ^{-1}, 10 mg ℓ^{-1} and 1 mg ℓ^{-1}, and C10 at 100 mg ℓ^{-1}, 50 mg ℓ^{-1}, 10 mg ℓ^{-1} and 1 mg ℓ^{-1}. A combined solution (1:1, v:v) of ABA + AMO 1618 was also tested. The concentrations of ABA and AMO 1618 in the solution were the same, and the mixed solution was tested over the range 10 mg ℓ^{-1}, 1 mg ℓ^{-1}, 0.1 mg ℓ^{-1} and 0.01 mg ℓ^{-1}.

After 4 to 6 weeks of incubation in a growth room, set at LD photoperiods at 25°C, the fresh weights and lengths of the shoots were measured and the mean values plotted against growth regulator concentration to give dose-response curves.

For the dose-response studies a minimum of 20 shoots were used per treatment, and in the case of AMO 1618, ABA + AMO 1618, C5, C10 and GA_3 treatments, the experiment was repeated twice, and four times in the case of the ABA treatments.

The effect of the presence of various concentrations of ABA (10 mg ℓ^{-1}, 1 mg ℓ^{-1}, 0.1 mg ℓ^{-1} and 0.01 mg ℓ^{-1}) in medium 1 was also tested, using 20 shoots and repeating the experiment twice.

2.9.5.5 The effect of ABA on the starch and sugar content of shoots

The sugar and starch content of aseptic shoots cultured on M2, containing ABA at either 10 mg ℓ^{-1}, 1.0 mg ℓ^{-1}, 0.1 mg ℓ^{-1} or 0.01 mg ℓ^{-1}, or GA_3 at 10 mg ℓ^{-1}, was determined.

Sugar and starch analysis was on shoot tissue used in the experiments described above (section 2.9.5.4). Total soluble sugars and starch content was analysed as described in section 2.3.3. For each concentration, four replicates of 10 shoots were analysed.

2.10 STATISTICS

Statistical use and interpretation were based on Sokal and Rohlf (1969).

In general, for each experiment at the treatment level, the means and the standard error of the mean (S.E.) were calculated as based on the number of replicates used. In some cases, where each replicate was composed of a number of items, e.g. in bioassays, the mean and its associated S.E. were also calculated for each replicate or experimental unit.

An analysis of variance (ANOVA) was used for the statistical separation of the major differences between means, and this analysis was performed on a Burroughs 3660 desk calculator using a prepared programme or on a Burroughs B6700 computer using a Fortran computer programme from the Sokal and Rohlf statistical package (Sokal and Rohlf, 1969). The complexity of the analysis used, one way, two way or rested analysis of variance, was dependent on the nature of the experiment in question.

In the case of the bioassay data, no attempt was made to use simultaneous interval estimation techniques (e.g. least significant difference, LSD) to distinguish which zones on a chromatograph were significantly different from the controls and from each other. The use of multiple comparison tests (MRT), such as Duncans MRT, Dunnetts MRT, and LSD tests such as Scheffes, Fishers, SNK and Tukeys, were considered to be inappropriate for the bioassay study, based on the arguments put forward by Chew (1976a,b, 1977, 1980), Sokal Rohlf (1969), Petersen (1977) and Baker (1980), and because of the complex nature of the analysis due to the use of solvent run chromatographs as controls. Instead of one control, against which all other R_F sections could be tested (for example in Dunnetts MRT), there was one control (an R_F section from the control chromatograph) for each R_F section from the extract or test chromatograph. This was further complicated by the

use of one control chromatograph for every three test or extract loaded chromatographs. Therefore, there were, in the case of test R_F sections, three replicates, each of which had differing variances and only one control R_F section. This made the use of a paired t test, or a modified t test as used by Fraser (1980), inappropriate for this study.

The null hypothesis Ho: that all means are equal (i.e. the bioassay response with each R_F section was the same), was tested for some chromatographs using ANOVA. It was accepted that when certain aliquots of extract were assayed, significant differences between the R_F sections on a chromatograph existed, and when standard errors of the mean overlapped, it was assumed there were little or no differences between the R_F sections being compared.

In order to assess the inhibitory activity of the inhibitor β fraction or zone, the area of the histogram columns making up the inhibitor β zone (R_F sections 8 to 13) was calculated. Any statistical differences between the area calculated for replicates, within harvests and between harvests, was analysed by one way and/or nested analysis of variance.

CHAPTER 3

RESULTS

3.1 STUDIES ON ENDOGENOUS LEVELS OF ABSCISIC ACID

An investigative approach, that can be used to determine whether ABA has a regulatory role in the induction of bud dormancy, is to quantify the amounts present in leaves and buds during induction. Changes in ABA can then be correlated with the onset of bud dormancy.

3.1.1 Identification of Abscisic Acid in Leaf and Apical Extracts of *Alnus viridis*

Extracts of leaves and buds were prepared such that there was a free ABA fraction and a "bound" ABA fraction. Both fractions were obtained from the same sample. The free ABA fraction was obtained from the ether-soluble fraction, and the "bound" ABA fraction was obtained from the aqueous fraction remaining after ether extraction. This remaining aqueous fraction was hydrolysed with alkali (pH 11) to release any bound or conjugated ABA as free acid, which was then re-extracted into ether. During further purification of the two fractions, it was found that the fractions could not be loaded on to TLC plates unless slurried with PVP. The brown gum-like residue that remained after the ether fractions had been taken to dryness was much reduced in volume and almost colourless following PVP treatment. The obvious reduction in dry weight made for simpler and more efficient thin layer chromatography.

The location of ABA on chromatographs was by comparison with the R_F of the mixed isomers of authentic ABA. A zone corresponding to the R_F of ABA marker spots was eluted and used for derivatisation of methyl abscisic acid (Me-ABA) and gas chromatography.

In all cases, gas chromatography of the purified extracts showed the presence of a peak with a retention time (7.74 min on OV 17) which was identical with cis, trans, Me-ABA (c,t, Me-ABA). A much smaller second peak corresponding to trans, trans, Me-ABA (t,t, Me-ABA)

(retention time 9.25 min on OV 17), was present in some extracts. Typical assay results obtained with gas chromatography are illustrated in figure 2A. The c,t, Me-ABA peaks represent approx. 100 pg quantities of ABA. Besides the occurrence of these two peaks, there were also several extraneous peaks present (figure 1). None of these peaks was identified and their interference with the c,t, Me-ABA peak was assumed to be minimal. There were fewer extraneous peaks in leaf extracts compared with apical extracts (figure 2).

Confirmation of the identity of the putative c,t, Me-ABA peak was obtained by co-chromatography with a known amount of authentic ABA standard. In all cases, the height of the c,t, Me-ABA peak was as expected and no other modification in the shape of the peak occurred. This is shown in figure 2B, where the co-chromatography assay profiles of the four types of extracts are illustrated.

Further confirmation was by chromatography on a range of stationary phases with differing polarities; SE 30, OV 1 and OV 210, in addition to OV 17. The response of the four columns to injections of mixed isomers of authentic ABA is illustrated in figure 3. The retention times for c,t, Me-ABA on the supplementary columns were 5.50, 3.33 and 2.83 min for SE 30, OV 1 and OV 210, respectively (Table 3.2). For all types of extract, there was a peak which co-chromatographed with c,t, Me-ABA. A typical set of assay results obtained on the three additional columns, with the four different extracts, is shown in figure 4. The authenticity of the putative c,t, ABA peak on the supplementary columns was also checked by co-chromatography. On no occasion was the c,t, Me-ABA resolved beyond a single component when co-chromatography with mixed isomers of authentic Me-ABA was attempted. An indication of the purity of the c,t, Me-ABA peak was reflected in the agreement between the quantification achieved on the four columns. Quantification of samples typical of the four types of extracts is presented in Table 3.1. In view of the wide range of adsorption and retention properties of the different stationary phases and columns, small discrepancies and errors were expected. However, in the case of two apical extracts, the estimate obtained on the OV 17 column was at variance with the estimates from the other three columns. The estimates for these extracts, no. 17 and no. 18, are listed in Table 3.1. As agreement between estimates was achieved on three columns, it was assumed that the amount of c,t, Me-ABA present in the sample

Table 3.1 Abscisic acid levels in leaf and apical extracts of *Alnus viridis* seedlings as determined on four stationary phases. Values represent nanograms per extract.

Stationary phase	Leaf free ABA	Leaf bound ABA	Apical free ABA	Apical bound ABA	Apical # 17 free ABA	Apical # 18 free ABA
OV 17	239.3	53.1	68.3	395.6	643.7	428.3
OV 1	244.9	53.9	69.6	359.6	181.3	229.6
OV 210	234.3	51.5	69.6	362.6	191.6	229.6
SE 30	236.5	53.1	65.2	362.6	181.3	215.2

was the average of these three columns only.

The presence of a peak, with a retention time similar to t,t, Me-ABA, was also detected in all extracts when these were chromatographed on the supplementary columns. With these three columns, the heights of the t,t, Me-ABA peaks, relative to the c,t, Me-ABA peaks, were increased when compared to the relative heights obtained on 3% OV 17. In some apical extracts, the relative heights of the c,t, Me-ABA and t,t, Me-ABA peaks were reversed, with the c,t, isomer peak being smaller than the t,t, peak. This lack of agreement is illustrated in figures 4 and 5, in which the heights of the peaks suggest the presence of only a trace amount of t,t, Me-ABA in profiles obtained with OV 17 and OV 210, but increased quantities as represented by the peaks obtained with columns OV 1 and SE 30. This suggested the possibility that some conversion between the isomers occurred during the time between analysis on the supplementary columns. However, as shown in Table 3.1, there was good agreement between columns with respect to the quantity of c,t, Me-ABA estimated by peak height measurement, and repeat analysis of a selected number of samples on OV 17 following the analysis on all three supplementary columns confirmed that isomerization between the two isomers had not occurred. From the analysis of results of co-chromatography with authentic ABA (figure 5), it was found that on OV 1, where large t,t, Me-ABA peaks were obtained, the shape of the peak was as expected. Similarly on OV 17 and OV 210, where only trace amounts of t,t, Me-ABA were detected, co-chromatography resulted in single peaks only. However, co-chromatography on the 10% SE 30 stationary phase showed that this putative peak was not t,t, Me-ABA and that there were at least two components present in the peak.

The retention time on this column was 7.50 min, which was noticeably different to the retention time of authentic t,t, Me-ABA (7.10 min). The profiles obtained for a leaf extract, before and after co-chromatography, are shown in figure 5. The modified shape of the putative t,t, Me-ABA peak upon co-chromatography, with authentic ABA isomers, was clearly visible with all extracts chromatographed on the SE 30 stationary phase. In contrast, on the other three columns, only a few seconds (±2) separated the authentic t,t, Me-ABA peak from the putative peak. The closeness of the retention times did not allow the peaks to be resolved beyond a single component when co-chromatographed, and therefore, gave the impression that a measurable quantity of t,t, Me-ABA was present, especially when extracts were chromatographed on OV 1. In view of this problem, with interfering substances, the quantification of the t,t, Me-ABA peak was not attempted, although it was accepted that trace quantities (usually less than 10 pg per injection) were probably present in all extracts. Where quantities greater than 10 pg per injection were shown to exist from the analysis on OV 17, and were in agreement with the estimates from the analysis on SE 30, the heights of the peaks at the retention times of c,t, Me-ABA and t,t, Me-ABA were combined to estimate the c,t, Me-ABA present in the extract. It was assumed that the occurrence of the very small quantities of t,t, Me-ABA in extracts had been a result of isomerization of the c,t, Me-ABA during the extraction and purification procedures. There was no evidence to suggest large quantities of t,t, ABA occurred within the plant tissues.

The chromatography properties, or more specifically the R_F, of phaseic acid are similar to ABA in the TLC system employed in this study (Dewdney, 1977), and therefore, it is possible that phaseic acid was the interfering compound. However, comparison of retention times (Table 3.2) and co-chromatography with authentic phaseic acid, on all four stationary phases, suggested that the second peak was not phaseic acid, although retention times on OV 17 and OV 1 were very similar.

The technique of U.V. isomerization was also used to verify the authenticity of the c,t, ABA peak. This was only partly successful in that a 1:1 ratio between cis, trans, and trans, trans, Me-ABA was not established (figure 6). Instead, the disappearance of the c,t, Me-ABA peak was accompanied by the appearance of several other extraneous peaks, in addition to the t,t, Me-ABA peak. This is not unexpected

Table 3.2 Retention times of derivatised, putative and authentic abscisic acid and derivatised phaseic acid on four different stationary phases. Times are in minutes.

Stationary phase	Authentic ABA		Putative ABA		Phaseic acid
	cis,trans	trans,trans	cis,trans	trans,trans	
OV 17	7.42	9.25	7.42	9.25	9.42
OV 210	4.00	4.17	4.17	2.83	3.42
OV 1	3.33	4.28	3.33	4.12	4.00
SE 30	5.50	7.10	5.50	7.50	6.42

according to Plancher (1980) and Dumbroff et al. (1979), especially if prolonged exposure to U.V. occurs. In this analysis, a time course of isomerization was not examined but all aliquots of samples were subjected to U.V. treatment for 1 hour before gas chromatography, as recommended by Lenton et al. (1971). It was assumed that transformation and breakdown of c,t, ABA occurred as proposed by Plancher (1980), and that a 1:1 ratio between c,t, and t,t, Me-ABA was not possible, or only occurred for a short time during the U.V. treatment.

Definitive identification of c,t, ABA was obtained using combined gas chromatography-mass spectrometry (GC-MS). The mass spectrum of the peak with the same retention time as c,t, Me-ABA showed major fragments and intensities at m/e 190, 162, 134, 125 and 91, which were in close agreement with those of authentic ABA (figure 7).

The comparison of retention times and co-chromatography on different stationary phases, U.V. isomerization, and GC-MS, therefore, indicated that in all extracts there was a compound with identical physical and chemical characteristics of c,t, Me-ABA. Only trace amounts of t,t, Me-ABA were present in extracts.

3.1.2 Abscisic Acid Levels and the Photoinduction of Bud Dormancy in *Alnus viridis*

The heights of seedlings maintained under LD's and SD's were measured at regular intervals (figure 8). Short days inhibited growth during the first week, and very markedly during the second week, after transfer to SD's. After 12 days of SD treatment, stem elongation had ceased and resting buds, as characterised by the appearance of red bud scales, were beginning to form. By day 21 of SD treatment, all buds

that remained on SD plants were assumed to be dormant. Transfer of six plants back to LD's for 4 weeks did not result in any further extension growth of the main axes of any of the plants, although some lateral branches on one plant were observed to be growing. This plant was, however, atypical in that excessive lateral branch growth had resulted in a very bushy, shrub-like appearance, which was uncharacteristic of the other plants in the study.

Samples of leaves and apices were taken for the determination of ABA content on the day of transfer from LD's to SD's, and on day 12 after transfer. The harvesting of plant tissue was executed during the latter half of the high light intensity period and the upper, fully expanded leaves were harvested separately from apices together with one-eighth expanded leaves. The ABA content of leaves and apices was determined by quantitative gas chromatography as outlined in section 3.1.

There was no effect of day length on the free ABA content of leaves or apices, but the ABA content per unit weight of leaf tissue was 39% of that found in apices (Table 3.3).

Table 3.3 Levels of free and bound abscisic acid in extracts from apices and leaves of alder seedlings grown under LD's (day 0) and after 12 days under SD photoperiods. Values represent ng kg^{-1} DW of tissue and are means ± S.E.'s from three replicate extracts. Each extract was from two plants.

	Day 0 (LD)		Day 12 (SD)	
	Free ABA	Bound ABA	Free ABA	Bound ABA
Leaf	172.0 ± 34.6	64.0 ± 6.5	203.1 ± 43.1	61.3 ± 12.4
Apices	456.6 ± 68.4	460.3 ± 25.0	502.5 ± 107.9	420.7 ± 69.1

The ABA content of leaves (approx. 188 ng per gram dry weight) and apices (approx. 480 ng per gram dry weight) was in the same order of magnitude and range as that reported by Phillips (1979, 1980), who worked with *Acer pseudoplatanus* seedlings. Appreciable quantities of esterified or bound ABA were present in leaves and apices harvested from LD's and SD's. However, daylength had no measurable effect on the content of bound ABA in leaves or apices. Apices contained seven times

more bound ABA than did the leaves. The ratio of free to bound ABA content of leaves and apices was not altered by the effect of daylength, although there was a difference in the ratio of free to bound ABA between the two types of tissue. An approximately 1 to 1 ratio of free to bound ABA was present in the apices, whereas the leaves were found to have a 3 to 1 ratio.

The data from this investigation suggested that the photo-induction of bud dormancy in alder under conditions of low night temperatures was not correlated with, or mediated through, changes in endogenous ABA levels.

3.2 STUDIES ON ENDOGENOUS LEVELS OF INHIBITOR β

The inhibitor β fraction was implicated in dormancy through studies on levels present in dormant and non-dormant tissues. Studies by a group at Aberystwyth led to the discovery of abscisic acid as the major inhibitory substance in the inhibitor β fraction that was responsible for the regulation of dormancy. In view of the results of the previous experiment in which the ABA content of leaves and apices was shown not to change in response to photoinduction of bud dormancy, a re-examination of the inhibitor β levels present in tissues during dormancy is vital. Bioassay methods represent the only means by which inhibitor β can be estimated.

3.2.1 Bioassay Systems

Two bioassay systems were employed to measure the inhibitor β content of tissues.

3.2.1.1 Wheat coleoptile section assay

The response of coleoptiles to the presence of ABA in the incubation medium is shown in figure 9. The shape of the dose-response curve was reverse sigmoid. With increasing concentration of ABA there is a marked decrease in extension growth, with maximum inhibition occurring at approx. 2×10^{-4}M ABA. The minimum detectable level of ABA was approx. 2×10^{-8}M, lower concentrations having no effect on coleoptile growth, although the shape of the curve suggests that the lower concentrations were promotory. The response of coleoptiles to ABA was

linear of the range 4×10^{-8}M to 4×10^{-6}M ABA. A series of standards was incubated at the same time as a series of bioassays, and from a random selection of typical dose-response curves shown in figure 9, no major changes in the shape and slope of curves were found, indicating that the bioassay material remained stable and was consistent throughout the analysis period.

The short chain fatty acids, C5 and C10, had a marked effect on coleoptile sections, but only at the highest concentration tested (1000 mg ℓ^{-1}) (figure 10). At the lower concentrations C5 did not have any inhibitory effect on growth, whereas for C10 there was some evidence of an inhibitory effect. However, the inhibitory effect was not comparable to ABA at equivalent concentrations on a weight basis. For example, coleoptile sections, in the presence of 100 mg ℓ^{-1} ABA, were strongly inhibited and their growth increment was 28.5% of the control, whereas in the presence of C10, the growth increment was 77.4% of the control. At the highest concentration (1000 mg ℓ^{-1}), both C5 and C10 were toxic to coleoptile sections and all sections were dead, whereas ABA reduced coleoptile extension growth to 17.6% of the control. Therefore, the concentration range over which the short chain fatty acids showed no effect to maximum (and toxic) effect was approximately a single order of magnitude, whereas ABA's range was over several orders of magnitude. The dose-response relationship for the short chain fatty acids was indicative of an arithmetical effect, in contrast to ABA's logarithmic effect on coleoptile section growth.

The response of coleoptiles to separate R_F sections of control (solvent run) chromatographs is shown in figure 11. Each R_F section was assayed with 10 coleoptiles. The histograms indicate that the response of coleoptiles to separate R_F sections of a control chromatograph resulted in growth promotion or growth inhibition. Although statistical analysis (two-way ANOVA, Table 3.4) of the data obtained from the first chromatograph shown in figure 11, revealed that there were significant differences between R_F sections ($0.01 < p < 0.05$), the response was not always repeatable. For example, R_F sections 4 and 5 of the chromatographs illustrated in figure 11 are shown to be both inhibitory and promotory to coleoptile growth. This unpredictable nature of the response was found to be a result of an interaction between the chromatography paper and the solvent used to develop the chromatograph.

Table 3.4 Two-way ANOVA of coleoptile assay on an unwashed solvent developed chromatograph (figure 11) involving 15 R_F sections (rows) and 10 coleoptiles per R_F section (columns).

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Coleoptiles (columns)	9	100.2	11.15	0.3506
R_F sections (rows)	14	880.4	62.89	1.9807*
Error	126	00.7	31.75	
Total	149	4 981.3		

The bioassay of a blank chromatograph (i.e., a sheet of chromatography paper that had been sectioned into 15 zones but had no other manipulation) resulted in a histogram as shown in figure 12. (Although the chromatographs illustrated in figure 12 were not run in solvent, an R_F scale has been included. This is to indicate that all chromatography sheets had corresponding tops and bottoms. Chromatographs were developed in the same direction as the machine direction (during their manufacture), which was indicated on the carton, and this "marker" was used to designate top and bottom for each chromatograph.) The results shown in figure 12 revealed that no R_F sections were significantly different from each other or from the water control (Fs = 1.422). However, most R_F sections were inhibitory to coleoptile growth relative to the water control. This suggested that the added presence of the developing solvent resulted in some of the R_F sections being significantly inhibitory to coleoptile growth (figure 11 and Table 3.4). The Fs statistic shown in Table 3.4 is barely significant at the 5% level (Fs = 1.9807 versus 1.9588), and the inhibitory response was not significant on all chromatographs. For example, the Fs statistic for chromatograph 3, illustrated in figure 11, was 1.477. However, the unpredictable nature of the response, together with the fact that there were significant differences between some R_F sections on some chromatographs, raised the question of the acceptability of using such a system for the detection and determination of inhibitors in plant extracts.

In order to improve the system, chromatographs were pre-washed in 80% MeOH for 3 hours and then air dried. The "washing" procedure was repeated a further two times prior to their being developed in the isopropanol/NH_3/H_2O solvent. The same solvents were used, but isopropanol and water were redistilled a second time before use. Bioassays of the pre-washed chromatographs are shown in figure 13. Significant differences between R_F sections were still present (Table 3.5), although washing substantially reduced the inhibitory effect and restricted it to a zone near the solvent front. Different batches of chromatography paper responded similarly (results not shown). Using Litmus paper, no differences between the pH's of different R_F sections could be detected.

Table 3.5 Two-way ANOVA of coleoptile assay on a washed, solvent-developed chromatograph (figure 13) involving 15 R_F sections (rows) and 10 coleoptiles per R_F section (columns)

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Coleoptiles (columns)	9	175.6	19.5	0.5615 n.s.
R_F sections (rows)	14	889.8	63.6	1.8313 *
Error	126	4 376.0	34.7	
Total	149	5 441.4		

An aqueous solution of a blank extract that had been through the extraction and fractionation steps as for plant extracts, but without the plant tissue, was found to have no significant effect on the growth of wheat coleoptiles (Table 3.6). The blank extract corresponded with the acid ether extract of plant material before chromatography and was taken up in 2 ml of water before assay. The growth of coleoptiles in water was 41.9 cm and 42.1 cm in the aqueous solution of the blank extract residue. Similarly, the dried residue of a few drops of the MeOH:Ether (1:1) solution had no effect on coleoptile growth. Washed chromatographs of blank extracts, or aliquots of blank extract, and washed chromatographs loaded with the equivalent number of drops of MeOH:Ether solution used to load the plant extracts, responded similarly

Table 3.6 The effect of blank extract and MeOH:Ether residues on the growth of wheat coleoptiles. Values are means ± S.E.'s of three replicates, each replicate consisting of 20 coleoptiles.

Residue	Coleoptile length (cm)
Blank extract	42.1 ± 1.2
MeOH:Ether	41.2 ± 1.0
Water (Control)	41.9 ± 0.8

in the bioassay. These results suggested that the inhibition seen on chromatographs was mostly a result of chromatography rather than the solvents used during the extraction and purification procedures.

Chromatographs, therefore, were washed three times in 80% MeOH before use, and for every three chromatographs loaded with extract, one chromatograph was loaded with a few drops of MeOH:Ether solution and used as the solvent control. Ideally, the solvent control should have been on the same chromatograph as that loaded with extract. However, it was not possible to line load the desired aliquot of extract on to one half of a chromatograph without overloading the chromatograph. It was accepted that using every fourth chromatograph as a solvent control offered the best workable system. The system was also practical from the point of view of the workload involved in running a bioassay.

A statistical comparison between the data obtained from three test (loaded with extract), three control and three blank chromatographs revealed that there were significant differences between R_F sections within chromatographs, and between chromatographs treated differently (i.e., test, control and blank), but no difference between chromatographs within the same treatment (Table 3.7). The nested analysis of variance also indicated that the variance component at level 2, chromatographs (replicates) within treatments, was low (2.5%), and therefore, the replication of chromatographs (3) was adequate. There were no significant differences ($p > 0.05$) between coleoptiles within the same R_F section as indicated by the statistical analysis shown in Tables 3.4 and 3.5. Therefore, from the bioassay system employed, it was possible to detect differences between chromatographs treated differently.

Table 3.7 Nested ANOVA of coleoptile assay involving test, control and blank chromatographs (treatments, level 3), 3 chromatographs per treatment (level 2), 15 R_F sections per chromatograph (level 1) and 10 coleoptiles per R_F section (level 0).

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Treatments (level 3)	2	6 205.20	3 102.60	9.0407 **
Replicate chromatographs (level 2)	6	2 059.07	343.18	2.2420 n.s.
R_F sections (level 1)	126	19 286.53	153.07	3.6648 ***
Coleoptiles within R_F section (level 0)	1 215	50 746.73	41.77	

Variance Components:	Level	Var. Comp.	Per cent
	3	6.3512	10.50
	2	1.2674	2.09
	1	11.1301	18.39
	0	41.7669	69.02

However, for confirmation of all bioassay results, a second bioassay system (the lettuce hypocotyl) was used.

3.2.1.2 Lettuce hypocotyl assay

The ability of ABA to inhibit hypocotyl elongation in dark grown lettuce seedlings is illustrated in figure 14. The dose-response relationship was linear over the range 10^{-7}M to 5×10^{-5}M, and maximum inhibition of hypocotyl growth occurred at approx. 2×10^{-4}M. In contrast to wheat coleoptiles, lettuce hypocotyls did not respond to ABA concentrations lower than 5×10^{-7}M although the shape of the dose-response curve was still reverse sigmoid.

The response of lettuce hypocotyls to the presence of various concentrations of C5, C10, and IAA is shown in figure 15. The presence of C5 was inhibitory to hypocotyl growth, whereas C10 had no effect or was slightly promotory. This is in contrast to the response of coleoptile sections where C5 and C10 were found to be inhibitory.

At equivalent concentrations by weight, C5 was less inhibitory to hypocotyl growth than ABA. The response of lettuce hypocotyls to a range of concentrations of C5 was indicative of an arithmetical effect, with the difference between no effect and maximum effect being approximately one order of magnitude. At the highest concentration (1000 mg ℓ^{-1}), C5 was toxic to germinated lettuce seeds and all seedlings were dead after 72 hours of treatment, whereas at 100 mg ℓ^{-1} only a small amount of inhibition of hypocotyl growth was detected. Indole-3-acetic acid was also found to be inhibitory to hypocotyl growth (figure 15).

It was found by trial and error, that the maximum growth of lettuce hypocotyls was achieved by placing the germinated seeds on to a 4.0 cm filter paper disc, which had been moistened with 1.5 ml of water. The growth of hypocotyls, in petri dishes without the filter paper disc but with 1.5 ml or 2.0 ml of water, was erratic; the coefficient of variations (n=20) were 20 and 25 per cent, respectively. Furthermore, hypocotyls grew less (10.6 mm versus 12.0 mm) in the presence of 2 ml of water per petri dish. Therefore, the reference on water control for the lettuce hypocotyl assay was set as the growth obtained by culturing the germinated seeds on a 4 cm filter paper disc moistened with 1.5 ml of water. The use of 1.5 ml of water per petri dish was continued when individual R_F sections were bioassayed.

The response of lettuce hypocotyls to solvent control chromatographs (pre-washed) is shown in figure 16. Two-way ANOVA (Table 3.8) confirmed that there were no significant differences ($p < 0.05$) between hypocotyls within the same R_F section, but there were significant differences between R_F sections within the same chromatograph. However, the degree of inhibition was negligible at R_F 0.5 to 0.8. At or near the solvent front (R_F's greater than 0.85), hypocotyl growth was promoted. This was in contrast to the response of wheat coleoptile sections, which were inhibited in the presence of this zone of the chromatograph.

Statistical analysis, 3 level nested ANOVA, revealed that a significant difference between test (extract loaded) chromatographs and control (solvent run) chromatographs as well as between R_F sections within chromatographs, could be detected using the bioassay system (Table 3.9).

Table 3.8 Two-way ANOVA of hypocotyl assay on a washed, solvent-developed chromatograph (figure 16) involving 15 R_F sections (rows) and 16 hypocotyls per R_F section (columns).

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Hypocotyls (columns)	15	238.1	15.87	0.1303 n.s.
R_F sections (rows)	14	3 248.4	232.03	1.7364 *
Error	210	28 061.8	133.0	
Total	239	31 548.3		

Table 3.9 Nested ANOVA of hypocotyl assay involving test, control and blank chromatographs (treatments, level 3), 3 chromatographs per treatment (level 2), 15 R_F sections per chromatograph (level 1) and 16 hypocotyls per R_F section (level 0).

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Treatments (level 3)	2	111 173.81	55 586.90	65.1100 ***
Replicate chromatographs (level 2)	6	5 122.43	853.74	0.41835 n.s.
R_F sections (level 1)	126	257 130.72	2 040.72	13.34661 ***
Hypocotyls per R_F section (level 0)	2 025	309 625.38	152.90	

Variance Components:	Level	Var. Comp.	Per cent
	3	78.7502	22.84
	2	-4.9457	-1.43
	1	117.9884	34.30
	0	152.9014	44.36

There were no significant differences between chromatographs treated similarly (replicate chromatographs), and the variance component (-1.4%) at this level of sampling confirmed that three replicates were adequate when comparing test and control chromatographs.

3.2.2 Seasonal (Autumn) Variation in the Inhibitor β Content

During the autumn period (March, April, May) there is a reduction in natural day length from 14 hours to 9 hours. Shoot extension growth of *Alnus glutinosa* during this period was observed to slow down and finally cease. At the time of the first harvest, most apical buds were considered to be growing since new leaves were emerging at the apex and red bud scales were absent. However, some lateral buds appeared dormant (red bud scales) and these were not harvested. At the beginning of April, most buds were developing red bud scales and there were very few buds with new emerging leaves. By May, all buds were dormant. Visual signs of leaf senescence, e.g. loss of chlorophyll and yellowing, were absent in leaves during the harvest period and no abscission of leaves had occurred. The level of growth inhibitors in extracts of leaves and apical buds was followed during three successive autumn periods; 1977, 1978 and 1979. The leaves and apical buds were harvested at approximately 3 week intervals.

All extracts were fractionated by paper chromatography using isopropanol/NH_3/H_2O (10/1/1) as the developing solvent, and the appearance of a typical solvent run chromatograph is illustrated in figure 17. Each chromatograph was divided into 15 equal R_F sections and these were numbered from the "bottom". Under visible light, a green band (chlorophyll?) was present at R_F 0.7 to 1.0. A brown pigment remained at the application zone, whereas the presence of a yellow substance was observed in the next two R_F sections (R_F 0.0 to 0.13). Inspection of the paper chromatographs, under long wave U.V. light, revealed the presence of several fluorescing and light absorbing bands in the zone R_F 0.4 to 0.9 as well as in the rest of the chromatograph. The degree of fluorescence and light absorption varied between the bands on the chromatograph. However, no major differences in the overall pattern of fluorescing and light absorbing bands was observed between similar extracts, although for apical and leaf extracts there was a difference in the degree of fluorescence and light absorption observed.

After paper chromatography, the leaf and apical extracts were bioassayed.

3.2.2.1 Autumn extracts 1977

Leaves and apices were harvested on five occasions during the autumn of 1977; March 7 (Harvest One, H1), March 25 (Harvest 2, H2), April 15 (Harvest three, H3), April 28 (Harvest four, H4) and May 15 (Harvest five, H5). The extracts were prepared as outlined in section 2.3.1.

(a) Leaf extracts, Wheat coleoptile assay. For each extract the equivalent of 0.25 g dry weight (DW) of leaf material was chromatographed and bioassayed. An aliquot of 0.25 g DW was used since this gave a response comparable with 10^{-6}M to 10^{-5}M ABA. Bioassay results obtained with the wheat coleoptile section assay for each of the five harvests H1, H2, H3, H4 and H5 are given in figures 18, 19, 20, 21 and 22, respectively.

Most R_F sections of test chromatographs were inhibitory to coleoptile growth, and little or no growth promotion was observed. Relative to the controls very little growth activity (promotion or inhibition) was detected in the first half of the chromatographs, R_F 0.0 to 0.5. All growth activity was confined to the limits of growth achieved by the assay of corresponding control R_F sections or the growth in the water (reference or internal) control. At all harvest dates no appreciable auxin activity was detected in the region R_F 0.2 to 0.4, which is the reported R_F of IAA.

The second half of the chromatographs from the first two harvests (figures 18 and 19) show two distinct zones of inhibition. The first was at the solvent front (R_F (0.87 to 1.0) and was assumed to be mostly due to the paper-solvent effect (described in section 3.2.1.1), although in some cases, some inhibition beyond that of the control was detected. A second zone of inhibition was most evident in the region R_F 0.5 to 0.9. This corresponded with the inhibitor β zone of Bennet-Clark and Kefford (1953). Within this zone, a peak of inhibition was detected in most chromatographs but the particular R_F at which the peak occurred varied, not only between harvest dates but also between extracts within the same harvest date. For example, in figure 19 the peak in inhibitory activity did not correspond between samples, although the degree of

inhibition associated with each peak did. The failure of the peak of inhibition to occur in the same R_F section in successive chromatographs was also found when authentic ABA was chromatographed. Spot checks in which an aliquot of authentic ABA was loaded on to a chromatograph and bioassayed after development in solvent, revealed that the peak of inhibition occurred anywhere between R_F 0.6 to 0.9, although it was mostly confined to only two successive R_F sections. Results of the spot checks, when made, are indicated in the figures as a horizontal line representing the R_F of authentic ABA. Therefore, results illustrated in figures 18, 19, 20, 21 and 22 indicate that both known (authentic ABA) and unknown (inhibitor β fraction of extracts) substances, chromatographed on different sheets of paper at different times, failed to appear in the same sections in successive chromatographs. However, replicates, in general, behaved similarly.

No attempt statistically was made to determine which R_F sections within the β fraction were significantly different from the controls or from each other (see section 2.10). Instead, the total amount of inhibition that occurred over the whole of the inhibitor β zone (0.5 to 0.8) was assessed for each extract at all harvest dates. The total amount of inhibition was assessed as being the area of the histogram outlined by the response to the water control and the test chromatograph for R_F sections 8 through to 13 inclusively. The area was calculated by summing the area of inhibition of each individual R_F section of the inhibitor β zone. Over the harvest period, there was an increase in total area of inhibition of the β zone (figures 17-21). The increase was gradual but by the end of April (H3) a marked increase had occurred. At the final harvest (H5), the increase in the area of inhibition over that found for H1 extracts , was a result of an increase in all R_F sections of the β zone. Initially, for example H3 extracts, most of the increase was confined to two R_F sections. This suggested that more than one inhibitory substance was responsible for the increase in total inhibition. To some extent, the bioassay results of chromatographs loaded with standards of authentic ABA supported this possibility (figure 23). Although a distinct ABA peak was observed in histograms representing the assay of quantities of ABA greater than 0.528 ng, the application of increasing amounts of ABA to chromatographs resulted in an increase in the number of R_F sections showing inhibition as well as as increase in the degree of inhibition within the R_F sections of the

original peak. This response is similar to that observed for the serially harvested extracts. However, the change in shape of the area of inhibition of histograms, of the serially harvested extracts, did not correspond with the pattern or change in shape of the area of inhibition observed with increasing amounts of ABA.

The dose-response relationship for two leaf extracts was also studied; an extract from the first harvest (sample #2 in figure 18) and from the last harvest (sample #1 in figure 22). A dilution series of chromatographs representing the equivalent of 0.025, 0.125, 0.25 and 0.315 g DW of leaf material (designated one-tenth, ½, 1 and 1½ dilutions, respectively) was bioassayed. The chromatography of dry weight equivalents greater than 0.375 g was not possible because of the presence of chlorophyll-like residue in the extracts. Substantial quantities of this residue made the loading process difficult and resulted in poor resolution when these chromatographs, which were visibly overloaded, were developed.

The histogram results of the dilution series for the H1 and H5 extracts are shown in figures 24 and 25, respectively. For both extracts, an increase in the aliquot of extract bioassayed, resulted in an increase in the inhibitory activity of the inhibitor β fraction. A one-tenth dilution of extract resulted in R_F sections having inhibitory activity that was equal or comparable to that of the control sections. A half dilution of extract was only slightly more inhibitory, whereas a sharp increase in inhibitory activity resulted when 0.25 g or more of leaf material was assayed. A region of growth promotory activity, R_F 0.0 to 0.4, was detected on chromatographs loaded with the equivalent of 0.375 g DW of leaf material from H5 (figure 25). However, for other chromatographs of the dilution series, growth promotion was not observed at this R_F zone or any other zone of any chromatograph. At all dry weight equivalents, except for the 1½ dilution, there was more inhibitory activity in H5 extract than H1 extract. For the 1½ dilution, the total amount of inhibition found in the inhibitor β zone was approximately the same for the two extracts. However, a comparison between the two extracts was complicated by the presence of more chlorophyll-like residue in the H1 extract, and as a result the developed chromatographs were overloaded. The shape of the resultant histograms reflected this fact. Overloading also restricted the range over which serial dilutions could be made, and a range similar to that

of authentic ABA was not studied. Nevertheless, the increase in inhibitory activity of the inhibitor β fraction of extracts (figures 24 and 25) and that of authentic ABA (figure 23) was not parallel. This was expected in view of the crude nature of the extracts.

The results suggested that the increased inhibitory activity of the β fraction of serially harvested extracts was the result of an increase in several inhibitors, and not the disappearance of a promoter or promoters co-chromatographing in this region.

A comparison of the total inhibition found within and amongst harvests was examined by nested analysis of variance. From this statistical analysis (Table 3.10) it was found that there was a significant difference between harvest dates but no significant differences within harvest dates. The analysis also indicated that the number of replicates used per harvest date was adequate, and that the increase in total inhibition (i.e. inhibitory activity of the inhibitor β fraction) observed over the harvest period was statistically significant ($p < 0.001$).

Table 3.10 Nested ANOVA of coleoptile assay of serially harvested leaf extracts (1977) involving five harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 18, 19, 20, 21 and 22.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	4	31 637.66	7 909.42	90.228 ***
Amongst extracts within dates	10	876.58	87.66	0.4016 n.s.
Extracts (error)	30	6 549.1	218.30	
Total	44	39 063.37		

The data from the histograms, therefore, suggested that there was an increase in the inhibitor β content of serially harvested leaf material over the autumn period. The increase in the inhibitory activity or content was gradual and was due to more than one inhibitor.

b. Leaf extract, Lettuce hypocotyl assay. The results of the lettuce hypocotyl assay are shown for all dates (H1, H2, H3, H4 and H5) in figures 26, 27, 28, 29 and 30, respectively. Most sections of the chromatographs were inhibitory to the hypocotyl growth, and growth promotion was detected in the first and last R_F sections only. The presence of this growth promotory activity was not detected by the wheat coleoptile assay. Most of the growth promotion at the solvent front (the last R_F section of the chromatograph) was the result of the interference of the paper-solvent interaction mentioned in section 3.2.1.2, whereas the promotory activity within the first R_F section, which included the origin plus the application zone, was the result of extract-derived activity. There was no major change in the amount of growth promotion that occurred within the first R_F section over the harvest period. No other growth promotion, relative to the limits of growth on control chromatographs or the water control, was found in any of the extracts.

Two distinct zones of growth inhibition were found on chromatographs of leaf extracts. The first zone occurred at R_F 0.2 to 0.4 and was most evident on chromatographs of extracts from the later harvests (figures 29 and 30). The R_F zone was the same as that reported for IAA. A peak of inhibition occurred within one section of this zone, either the 3rd or 4th R_F section, but no consistent pattern between extracts of the same harvest or between harvests was noted. Over the harvest period, an increase in the amount of inhibition within this zone had occurred. A comparison of replicate chromatographs of the same extracts, for example, histograms in figures 21 and 22, compared to those in figures 29 and 30, revealed that the inhibitory zone located at R_F 0.2 to 0.4 was mostly inactive, but occasionally slightly promotory, in the wheat coleoptile assay. In addition, hypocotyl growth was inhibited when germinated seeds were incubated on IAA solutions (figure 15), and therefore, it is possible that this zone of inhibition at R_F 0.2 to 0.4 was a reflection of some auxin-like activity.

On all chromatographs of extracts, a second zone of inhibition of greater intensity occurred at R_F 0.5 to 0.9. The R_F range corresponded to that of the inhibitor β zone of Bennet-Clark and Kefford (1953) and to the zone of inhibition found on replicate chromatographs bioassayed using wheat coleoptiles. Within the inhibitor β zone, a peak in inhibition occurred in two successive R_F sections. However, the

position of the two R_F sections varied between chromatographs, including chromatographs of extracts within the same harvest date. Nevertheless, a distinct peak in inhibition was evident in the zone R_F 0.6 to 0.8. The peak coincided with that detected by the wheat assay, and overall, the histogram data suggested that the wheat and lettuce assays were quantitatively similar in their response to the β zone.

A comparison between the quantitative response of the wheat and lettuce assays to the same inhibitor β fraction was made. The inhibition found in individual R_F sections of the β zone was expressed as equivalent ABA concentrations, although the bioassay of these R_F sections did not represent pure ABA solutions. The concentration estimates were obtained from the standard curves in figures 14 and 9 for the lettuce and wheat assays, respectively. For individual R_F sections of H1 and H5 chromatographs, the maximum amount of inhibition detected by the lettuce assay and expressed in concentrations of ABA was 4.5×10^{-6}M and 1.1×10^{-5}M, respectively (hypocotyl growth was 55% and 45% of the control growth, respectively), whereas for the wheat assay, the equivalent ABA concentrations were 8.1×10^{-7}M and 4.5×10^{-6}M, respectively. The estimates determined by the lettuce assay were expected to be higher than those determined by the wheat assay because the individual R_F sections were eluted and incubated in 1.5 ml of water compared to 2 ml. However, the estimates suggested that there was a quantitative difference between the response of the wheat and lettuce assays to the same inhibitor β zone, although both bioassays revealed that there was an increase in the inhibitory activity of the β zone over the harvest period.

As with the wheat assay, no attempt was made to find which R_F sections within the β zone were significantly different from the controls or from each other. The practice of assessing the total amount of inhibition in the β zone by summing the area of inhibition of each individual R_F section was continued. At the end of April (H3) there had been a small increase in the area of inhibition, and at the next two harvest dates (H4 and H5), a further increase was found compared to the area of inhibition found on chromatographs of H1 extracts. The increase in area of inhibition observed on chromatographs of H5 extracts was the result of an increase in inhibition in all R_F sections of the inhibitor β zone. However, most of the inhibition occurred at R_F 0.6 to 0.8 or in two successive R_F sections adjacent to

this zone.

The dose-response histograms obtained for standard quantities of authentic ABA are shown in figure 31. Relative to the controls, no inhibitory response was found when 0.053 µg was chromatographed and bioassayed. The chromatography and bioassay of 0.53 µg of ABA resulted in an inhibitory response in the zone R_F 0.73 to 0.87, i.e. within the "inhibitor β region" of the chromatograph. The application of increased amounts of ABA to chromatographs resulted in an increase in the area of of inhibition found within the R_F zone 0.70 to 0.87 (R_F sections 12 and 13). Most of the increase in the area of inhibition was confined to two successive R_F sections (numbers 12 and 13). In this respect, the change in shape of the area of inhibition did not correlate with the change in shape of the area of inhibition observed for the histograms of H1 and H5 extracts.

Lettuce assay results of the serial dilution at 0.025, 0.125, 0.25 and 0.375 DW of leaf material of H1 and H5 extracts are given in figures 32 and 33, respectively. An increase in the aliquot of extract bioassayed resulted in a large increase in the inhibitory activity of the β zone and a smaller increase in the inhibitory activity of the zone R_F 0.2 to 0.4. This trend was observed in both extracts, although for H1 extracts the chromatographs of aliquots representing 0.375 g DW equivalent of leaf material were overloaded and two distinct zones of inhibition were not seen. No growth activity beyond the limits of the controls was observed on chromatographs of aliquots representing 0.025 g DW equivalent of leaf material of H1 or H5 extract. The assay of extracts at 0.125 g DW equivalents resulted in some inhibition within the β zone. At the higher DW equivalents, 0.25 g and 0.375 g, a further increase in the area of inhibition within the β zone was observed, whereas no marked changes in growth promotory activity were found on chromatographs. The area of inhibition was greater for chromatographs of H5 extract than chromatographs of H1 extracts. For both extracts, the increase in the area of inhibition, with increasing aliquots of applied extract, was a result of an increase in the number of inhibitory R_F sections as well as an increase in the inhibitory activity of each R_F section. In this regard, the dose-response histograms did not parallel the dose-response histograms of authentic ABA. The dose-response histograms shown in figures 32 and 33 suggested that the increase in the area of inhibition was due to the increased

presence of more than one inhibitory substance. Similarly, the histogram data for the serially harvested extracts (figures 26, 27, 28, 29 and 30) suggested several inhibitory substances could be responsible for the increase in the area of inhibition of the β zone found on chromatographs of H4 and H5 extracts.

The areas of inhibition, within the β zone of chromatographs of the same and different extracts for each harvest date, were analysed by nested analysis of variance (Table 3.11).

Table 3.11 Nested ANOVA of hypocotyl assay of serially harvested leaf extracts (1977) involving five harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 26, 27, 28, 29 and 30.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	4	184 312.16	46 078.04	110.23 ***
Amongst extracts within dates	10	3 135.57	313.56	0.7501 n.s.
Extracts (error)	30	12 540.3	418.0	
Total	44	199 988.04		

The variation in the area of inhibition between extracts within harvest dates was non significant, and the very low variance component at this level indicated that replication of extracts within harvest dates was more than adequate. The variation in the area of inhibition between harvest dates was significant ($p < 0.001$). Therefore, the analysis of the histogram data, obtained from the lettuce hypocotyl assay, indicated that there was a significant increase in the area of inhibition, within the β zone, of chromatographs of serially harvested extracts.

The results of the two bioassays suggested that there was a significant increase in the inhibitor β content of leaves over the autumn period. The increase was gradual and may be due to more than one inhibitory substance. Only a small increase in total inhibitory

activity was found in leaves harvested at the end of April, when the majority of buds were already dormant.

(c) Apical extracts, Wheat coleoptile assay. For each extract, the equivalent of 0.2 g DW of apical material was chromatographed and bioassayed. The maximum inhibitory response of coleoptiles to R_F sections from chromatographs of H1 and H5 extracts was equivalent to the coleoptile response to 10^{-6}M ABA. The results of the wheat coleoptile assay are shown in figures 34, 35, 36, 37 and 38 for harvests H1, H2, H3, H4 and H5, respectively. At each harvest, apical extracts were mostly inhibitory to coleoptile growth. No R_F sections were promotory to coleoptile growth beyond the limits of the controls. In general, most R_F sections from the first half of the chromatograph R_F 0.0 to 0.5 were inactive in the coleoptile assay and neither a promotory nor an inhibitory response, beyond that of the controls, was observed in this zone.

In the second half of the chromatographs, a zone of inhibition was evident on all chromatographs at R_F 0.5 to 0.9, which corresponded with the inhibitor β zone of Bennet-Clark and Kefford (1953). Within the β zone, a broad peak of inhibition was observed. The peak consisted of three successive R_F sections, and its position varied between extracts from the same harvest date and between extracts from different harvest dates, but was consistent between replicate chromatographs of the same extract.

The area of inhibition of the β zone was determined and accepted as an estimate of the total inhibitory activity. Chromatographs of extracts from the later harvests H3, H4 and H5 showed an increase in the area of inhibition relative to the chromatographs of H1 extracts. The variation in the area of inhibition, between and within harvest dates, was analysed by nested ANOVA and the results of the analysis are shown in Table 3.12. No significant differences were found between the area estimates of replicate extracts within the same harvest date, but the differences in the area of inhibition between harvest dates were significant.

The increase in the area of inhibition was a result of an increase in the inhibitory response in all R_F sections of the inhibitor β zone. The peak of inhibition was more intense and broader on chromatographs of H5 extract (figure 38) compared with chromatographs

Table 3.12 Nested ANOVA of coleoptile assay of serially harvested apical extracts (1977) involving five harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 34, 35, 36, 37 and 38.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	4	13418.97	3354.74	13.562 ***
Amongst extracts within dates	10	2473.50	247.35	0.9816 n.s.
Extracts (error)	30	7559.99	251.99	
Total	44	23452.46		

of H1 extracts (figure 34).

The bioassay results of the serial dilution of H1 and H5 extracts are shown in figures 39 and 40, respectively. Only a narrow range of dilutions (0.02, 0.10, 0.20 g DW apical material) was tested as there was insufficient extract to fulfil the number of replicates needed for both bioassays. The H1 and H5 extracts assayed were the same as the extracts which are represented as the third histogram in figures 34 and 38, respectively. For each extract, a peak of inhibition within the β zone increased in intensity and width with each increase in aliquot of extract chromatographed. At the lowest dilution (0.02 g), the growth response to R_F sections was not markedly different from the response to R_F sections from control chromatographs, whereas a single R_F section (number 10) on chromatographs of H5 extract, assayed at dilution equivalent to 0.1 g DW of apical material, was found to be inhibitory. At the highest aliquot assayed, a zone of inhibition was present within the β zone and a distinct peak in inhibitory activity, which consisted of several successive R_F sections, was observed. The peak and/or area of inhibition within the β zone was greater for chromatographs of H5 extracts as compared to H1 extracts, when these extracts were assayed at a dilution equivalent to 0.1 and 0.2 g DW of apical material. The dose-response relationship observed for H1 and H5 extracts did not parallel that found for authentic ABA (histograms in figures 34 and 38 compared with histograms in figure 23).

The wheat assay data suggested that there was an increase in the inhibitory activity of the β zone; there was more inhibitory activity in the inhibitor β fraction of apical extracts harvested at the end of autumn than at the beginning of autumn. The apparent progressive increase in the inhibitory activity of the inhibitor β fraction of buds during the autumn was significant.

(d) Apical extracts, Lettuce hypocotyl assay. The lettuce assay results are shown in figures 41, 42, 43, 44 and 45 for harvests H1, H2, H3, H4 and H5, respectively.

The growth activity of the chromatographs was mostly inhibitory to hypocotyl extension. Growth promotion was confined to two R_F sections of the chromatograph; the first and last sections. However, no consistency in the activity of these two R_F sections was found between the extracts within the same harvest date, and no trend or change in promotory activity was observed between extracts of different harvests.

Two zones of inhibitory activity were detected on the chromatographs. One zone occurred at R_F 0.2 to 0.3 but was not present on all chromatographs, whereas a second zone of inhibition of much greater intensity than the first zone, occurred at R_F 0.5 to 0.8 and was present on all chromatographs. This second zone corresponded to the R_F range of inhibitor β. Within the β zone of a chromatograph, a peak in inhibitor activity was evident and this peak consisted of several successive R_F sections. The most inhibitory R_F section was not the same on all chromatographs but always lay between R_F 0.6 and 0.8. The peak of inhibition within the β zone was broader and more intense in chromatographs of H5 extracts (figure 45) than in chromatographs of H1 extracts (figure 41).

The bioassay results of the serial dilution of the H1 extract and H5 extract are shown in figures 46 and 47, respectively. The dose-response relationship was not identical to that of authentic ABA, although there was an increase in inhibitory activity with each increase in the aliquot of extract assayed. For both extracts, a dilution, equivalent to 0.02 g DW of apical material, resulted in no marked growth activity relative to the growth activity of control chromatographs. On chromatographs of H5 extract, assayed at the equivalent of 0.1 g DW of apical material, a single R_F section (no. 10)

was found to be markedly inhibitory to growth (figure 47). An inhibitory R_F section (no. 9) of less intensity was also detected on chromatographs of H1 extract (figure 46). At the highest aliquot chromatographed (the equivalent of 0.2 g DW of apical material, the R_F sections within the β zone, on chromatographs of H5 extract, were more inhibitory than corresponding R_F sections on chromatographs of H1 extracts.

A progressive increase in the inhibitory activity of serially harvested extracts, represented in figures 41, 42, 43, 44 and 45, was observed. The increase in inhibitory activity of the β zone was analysed statistically by nested ANOVA after determining the area of inhibition within the zone. The results of the analysis (Table 3.13) indicate that there were significant differences ($p < 0.001$) between the areas of inhibition estimated for the different harvest dates.

Table 3.13 Nested ANOVA of hypocotyl assay of serially harvested apical extracts (1977) involving five harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 41, 42, 43, 44 and 45.

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	4	89 663.11	22 415.78	50.000 ***
Amongst extracts within dates	10	4 514.99	451.50	1.1536 n.s.
Extracts (Error)	30	11 741.09	391.37	
Total	44	105 919.19		

Therefore, the increase in the inhibitory activity of the inhibitor fraction of apical extracts was significant. The statistical analysis also indicated that there had been adequate replication, and that replicate extracts within the same harvest date were not significantly different from each other.

The lettuce assay results suggested that the inhibitor content of buds increases progressively over the autumn.

The results of the wheat and lettuce assay indicated that, over the 1977 autumn season, there was a progressive increase in the inhibitor β content of leaves and apices of *Alnus glutinosa*. The increase was gradual but significant and appeared to involve more than one inhibitory substance. Apical tissue had a higher content of inhibitors than did leaf tissue when compared on a dry weight basis.

3.2.2.2 Autumn extracts 1978

Leaves and apical buds were harvested separately on three occasions during the autumn of 1978; 13 March, 14 April and 2 May, designated H1, H2 and H3, respectively. Natural daylength declined progressively from approx. 13.5 hours to approx. 9 hours during this period. Extracts were prepared for assay on wheat coleoptile sections on lettuce hypocotyls as outlined in section 2.3.1.

(a) Leaf extracts. An extract from the first (H1) and last (H3) harvest dates was assayed at serial dilutions equivalent to 0.025, 0.125 and 0.25 g DW of leaf material (designated d1, d2 and d3, respectively). On chromatographs of the lowest dilution (d1), no marked growth activity relative to the growth limits of the controls was detected by either the wheat assay (figures 48 and 49) or the lettuce assay (figures 50 and 51). Chromatographs of extracts at the highest dilution (d3) were overloaded and most R_F sections were inhibitory in the two bioassays. A peak of inhibitory activity within the inhibitor β zone was detected on chromatographs of H1 extract (figures 48 and 50) and H3 extract (figures 49 and 51) when assayed at d2. Because of the better resolution on the chromatographs, it was decided to assay all extracts using the equivalent of 0.125 g DW of leaf material. This was half the aliquot of extract assayed the previous year, and suggested that the inhibitor content of leaves of the 1978 season was higher than in leaves of the 1977 season. However, comparison is complicated by the troublesome presence of chlorophyll-like residue.

The results of the wheat assay are shown in figures 52, 53 and 54, representing extracts from H1, H2 and H3, respectively. The chromatograph pattern was similar to that found with extracts of leaves from the previous year. The growth response of coleoptiles to R_F sections from the first half of the chromatograph (R_F 0.0 to 0.5)

was similar to the response of coleoptiles to the corresponding control R_F sections or the water control, and in all cases, growth activity within the first half of the chromatographs was within the growth activity limits of the controls. Most R_F sections of the second half of the chromatographs (R_F 0.5 to 1.0) were inhibitory to coleoptile growth, but a distinct peak in inhibition was observed within the zone at R_F 0.6 to 0.7. There was more inhibitory activity within the inhibitor β fraction of H3 extracts (figure 54) than H1 extracts (figure 52) and this difference was significant ($p < 0.01$, Table 3.14).

Table 3.14 Nested ANOVA of coleoptile assay of serially harvested leaf extracts (1978) involving three harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 52, 53 and 54.

Source of variation	Degrees of freedom	Sum-of-squares	Mean squares	Fs
Amongst dates	2	7605.77	3802.89	11.4576 **
Amongst extracts within dates	6	1991.46	331.91	1.2511 n.s.
Extracts (Error)	18	4775.13	265.29	
Total	26	14372.36		

A second inhibitory zone at R_F 0.8 to 1.0 was found on some chromatographs (e.g. figure 52). Inhibitory activity within this zone was also more intense in H3 extracts than H1 extracts.

The lettuce assay results (figures 55, 56 and 57, representing H1, H2 and H3 extracts, respectively) were similar to the previous year. A growth inhibitory zone corresponding to the inhibitor β zone was found on all chromatographs. The inhibitory activity of the β zone of chromatographs of H3 extracts was significantly ($p < 0.001$) greater than the β zone of H1 extracts (Table 3.15). Two growth promoting zones were found on chromatographs, at R_F 0.0 to 0.2 and R_F 0.8 to 1.0. The first zone was not active in the wheat bioassay, whereas the second zone was inhibitory to coleoptile section growth. The growth promotion in these zones was greater than the corresponding zones on chromatographs of 1977 extracts.

Table 3.15 Nested ANOVA of hypocotyl assay of serially harvested leaf extracts (1978) involving three harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 55, 56 and 57.

Source of Variation	Degrees of	Sum-of-squares	Mean-squares	Fs
Amongst dates	2	91 583.0	45 791.5	135.790 ***
Amongst extracts within dates	6	2 023.7	337.3	0.2676 n.s.
Extracts (Error)	18	22 686.7	1 260.4	
Total	26	116 293.4		

The results from the two bioassays suggested that there had been a progressive increase in the inhibitory activity of the inhibitor β fraction of serial extracts of leaf material harvested during the period of bud dormancy development. The inhibitory activity of leaf extracts was greater than the previous year.

(b) Apical extracts. The equivalent of 0.2 g DW of apical material was bioassayed. The results of the wheat assay of H1, H2 and H3 extracts are shown in figures 58, 59 and 60, respectively. These results were similar to the lettuce hypocotyl assay results shown in figures 61, 62 and 63, which represent H1, H2 and H3 extracts, respectively. A major zone of inhibition occurred on all chromatographs at R_F 0.5 to 0.9. A distinct peak of inhibition occurred at R_F 0.6 to 0.8. A small increase in the area of inhibition within the β zone was observed for the series of chromatographs shown in the sequence; figures 58, 59 and 60 (wheat assay), and figures 61, 62 and 63 (lettuce assay).

Statistical analysis of the wheat assay data (Table 3.16) and the lettuce assay data (Table 3.17) showed that the increase was significant ($p < 0.05$). With the wheat coleoptile assay, a second zone of inhibition at R_F 0.25 to 0.35 was observed on some chromatographs, particularly those of H2 extracts. This zone was less intense than the inhibitory zone at R_F 0.5 to 0.9 and was not present in H3 extracts. The R_F sections of this second zone were not inhibitory in the lettuce hypocotyl assay.

Table 3.16 One-way analysis of variance of coleoptile assay of apical extracts (1978) involving three harvest dates (treatments) and three extracts per harvest (replicates). Data (areas of inhibition of β zones) are shown in figures 58, 59 and 60.

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Treatments	2	4 112.27	2 056.13	8.0158 *
Error	6	1 539.06	256.51	
Total	8	5 651.33		

Table 3.17 One-way analysis of variance of hypocotyl assay of apical extracts (1978) involving three harvest dates (treatments) and three extracts per harvest (replicates). Data (areas of inhibition of β zones) are shown in figures 61, 62 and 63.

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	
Treatments	2	2 497.54	1 248.77	6.1011 *
Error	6	1 228.08	204.68	
Total	8	3 725.62		

The results suggested that during the development of dormancy, there was an increase in the inhibitory activity of the inhibitor β fraction of apices. The increase was small but significant.

3.2.2.3 Autumn extracts 1979

Sampling was from the same trees as those used in previous years. Three harvests were made; on 5 March (designated H1), 13 March (H2) and 4 April (H3). The leaf and apical extracts were prepared as outlined in section 2.3.1.

(a) Leaf extracts. All extracts were assayed at the equivalent to 0.25 g DW of lead material. Initially, two extracts, one H1 and one H3, were assayed at serial dilutions equivalent to 0.025, 0.125 and

0.25 g DW of leaf material. The results of the wheat assay (figures 64 and 65; H1 and H3 extract, respectively) and the lettuce assay (figures 66 and 67; H1 and H3 extract, respectively) indicated that the second highest dilution assayed was most suitable, since the inhibitory response of coleoptiles and hypocotyls to R_F sections was approximately 50% of the maximum possible. Some inhibitory activity within the zone was present on chromatographs of extract equivalent to 0.125 g DW of leaf material. There was more inhibitory activity on chromatographs of H3 extract than H1 extract, when extracts were compared at the two highest dilutions.

The results of the assay of serially harvested extracts on wheat coleoptiles are shown in figures 68 (H1), 69 (H2) and 70 (H3). No growth promotory activity was detected on the chromatographs. The bioassay response in the region R_F 0.0 to 0.5, of chromatographs loaded with extract, was the same as the response to control chromatograph sections. A major zone of inhibition occurred at the same R_F as the inhibitor β zone of Bennet-Clark and Kefford (1953). The inhibitory activity within this zone peaked in successive R_F sections, the position of which varied for different extracts but was always within the R_F range 0.5 to 0.7. A second, less intense peak was also observed within the β zone and was located at R_F 0.8 to 0.93.

The within season changes in the inhibitory activity occurring at R_F 0.5 to 0.9 are shown in figures 68, 69 and 70. The total inhibitory activity, which was assessed by measuring the area of inhibition within the inhibitor β zone, increased progressively. The increase was due to an increase in the level of inhibition in all R_F sections, and was significant ($p < 0.01$, Table 3.18).

Changes in inhibitory activity of the β zone, as detected by the wheat assay, were confirmed by the assay on lettuce hypocotyls. The results for H1, H2 and H3 extracts are shown in figures 71, 72 and 73, respectively. The pattern of growth activity detected on chromatographs was similar to previous years. Most R_F sections were inhibitory to hypocotyl growth. A broad zone of inhibition was found in the first half (R_F 0.0 to 0.5) of each chromatograph. A peak in inhibition was centred at R_F 0.2 to 0.4, and its intensity varied between replicate extracts of the same harvest date. No marked

Table 3.18 Nested ANOVA of coleoptile assay of serially harvested leaf extracts (1979) involving three harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 68, 69 and 70.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	2	6 791.49	3 395.75	73.69 **
Amongst extracts within dates	6	276.49	46.08	0.3601 n.s.
Extracts (Error)	18	2 303.11	127.95	
Total	26	9 371.08		

differences in the inhibitory activity of this zone were observed between extracts of different harvest dates. No attempt was made to assess the total inhibitory activity within this zone of chromatographs.

A second, more intense zone of inhibition was distinguishable at R_F 0.5 to 0.9. Within this region, two peaks in inhibition were found. The first, and largest peak was located in the region R_F 0.5 to 0.8 and was prsent on all chromatographs, whereas the second, and smaller peak was centred at R_F 0.85 to 0.95 and was absent on some chromatographs. Changes in inhibitory activity of the two peaks occurred and are shown in figures 71, 72 and 73. Although the changes were small, a significant ($p < 0.05$, Table, 3.19) increase was found in the total inhibitory activity.

Table 3.19 Nested ANOVA of hypocotyl assay of serially harvested leaf extracts (1979) involving three harvest dates (level 2), three extracts per harvest date (level 1) and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 71, 72 and 73.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	
Amongst dates	2	10 884.31	5 442.16	13.1013 **
Amongst extracts within dates	6	2 492.34	415.39	1.4318 n.s.
Extracts (Error)	18	5 222.18	290.12	
Total	26	18 598.84		

The results obtained from the two bioassays suggested that, in leaves, the inhibitory activity of the β fraction progressively increased during autumn. The increase was small but significant.

(b) Apical extracts. All extracts were assayed at a dilution equivalent to 0.2 g DW of apical material. The results of the assay of R_F sections on wheat coleoptiles are presented in figures 74, 75 and 76 for H1, H2 and H3, respectively. All chromatographs were similar in their influence on coleoptile section growth. No growth promotion, relative to the controls, resulted from the assay of any of the R_F section and in general, little or no growth activity was detected on the first half of the chromatograph.

A zone of inhibition was present in the second half of the chromatograph, particularly at R_F 0.5 to 0.8. The inhibitory areas within the β zone of H1, H2 and H3 extracts were similar and no significant differences ($p > 0.05$) between extracts within the same or different harvest dates were found (Table 3.20).

Table 3.20 One-way analysis of variance of coleoptile assay of apical extracts (1979) involving three harvest dates (treatments) and three extracts per harvest (replicates). Data (areas of inhibition of β zones) are shown in figures 74, 75 and 76.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs	
Treatments	2	428.42	214.21	0.5378	n.s.
Error	6	2 389.8	398.3		
Total	8	2 818.2			

The wheat assay on the serially harvested apical extracts suggested that no change in the inhibitory activity of the β fraction of extracts occurred during autumn.

The results of the lettuce hypocotyl assay on serially harvested extracts (figures 77, 78 and 79 for H1, H2 and H3 extracts, respectively) were similar to those obtained with the wheat assay. Most R_F sections were inhibitory to hypocotyl growth. A major zone of inhibition was detected in the second half of the chromatograph at the same R_F as the

inhibitor β zone, R_F 0.5 to 0.8. Within this zone, inhibition was maximal in R_F sections at R_F 0.5 to 0.75 and was comparable to 10^{-5}M ABA. In this respect, the inhibitory activity of the apical extracts was greater than the inhibitory activity of leaves and of apical extracts from previous years.

Total inhibitory activity, assessed by measuring the area of inhibition within the β zone, was significantly less in H3 extracts when compared with H1 extracts (Table 3.21). The smaller area of inhibition in histograms of H3 extract was a result of a decrease in inhibitory activity in some R_F sections only, and not in all R_F sections of the β zone. The maximum inhibition in a single R_F section was still comparable to 10^{-5}M ABA.

Table 3.21 One-way analysis of variance of hypocotyl assay of apical extracts (1979) involving three harvest dates (treatments) and three extracts per harvest (replicates). Data (areas of inhibition of β zones) are shown in figures 77, 78 and 79.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	
Treatments	2	5 760.1	2 880.05	6.7376 *
Error	6	2 564.76	427.46	
Total	8	8 324.86		

An extract from the first (H1) and last (H2) harvest dates was assayed at serial dilutions equivalent to 0.02, 0.10, 0.2 and 0.35 g DW of apical material. The results of the wheat (figures 80 and 81 for H1 and H3 extracts, respectively) and the lettuce (figures 82 and 83 for H1 and H3 extracts, respectively) assays were similar. At all dilutions, no marked differences between the area of inhibition on the chromatographs of the two extracts were observed. Most R_F sections assayed, either on wheat coleoptile sections or lettuce hypocotyls, were inhibitory to growth, irrespective of the dilution at which the extract was assayed. However, maximum inhibition of growth was never achieved. At the lowest dilution, no marked growth respones beyond the limits of the controls were observed on chromatographs, whereas at the highest dilution assayed, a major zone of inhibition at R_F 0.5 to

0.9 was detected on chromatographs of both extracts. Inhibition within this zone was most intense at R_F 0.6 to 0.8. Intermediate dilutions show a progressive increase in inhibition within this zone. The dose-response relationship, however, did not parallel the dose-response relationship established for authentic ABA (figures 23 and 31, wheat and lettuce assays, respectively).

The bioassay data suggested the inhibitory activity of the inhibitor β fraction of apices did not change (wheat assay), or decreased (lettuce assay), during the autumn of 1979. The inhibitor content of apical buds was higher during 1979 than previous years.

3.2.3 Characterisation of Inhibitor β

Two apical extracts corresponding to H1 and H3, 1978, were used in the study. The inhibitor β fraction was isolated and prepared from aliquots equivalent to 0.25 g DW of apical tissue, as outlined in section 2.3.1. The acidic ether-soluble fraction was chromatographed with isopropanol/ammonia/water (10:1:1) and the zone, R_F 0.5 to 0.85, was eluted and rechromatographed with butanol/ammonia (5:1).

After development, a replicate set of chromatographs were sprayed with reagents for detecting phenolic compounds. Positive reactions occurred at R_F 0.05 to 0.12, R_F 0.53 to 0.6 and R_F 0.80 to 0.87 (figure 84). Within these R_F zones, the colour reactions were less intense on H3 chromatographs than H1 chromatographs.

The results of wheat coleoptile assay indicated that all R_F sections were inhibitory to coleoptile growth and that two major zones of inhibition, at R_F 0.3 to 0.5 and R_F 0.87 to 1.00, were present on the chromatographs (figure 85). The inhibitory activity at the solvent front was also present on control chromatographs, and therefore, was considered to be the result of solvent interference. There was no phenolic-positive reaction associated with the main inhibitory region, R_F 0.3 to 0.5. The chromatography zones, in which phenolic compounds were shown to be present, were only slightly inhibitory to coleoptile growth relative to the controls. The inhibitory activity at R_F 0.3 to 0.5 was more intense on chromatographs of H3 extract than on chromatographs of H1 extract. No other marked differences between chromatographs were observed.

The results of the lettuce hypocotyl assay confirmed that most of the inhibitory activity on chromatographs was confined to the R_F zone, 0.3 to 0.5, and that it was greater on H3 chromatographs than on H1 chromatographs (figure 86). No other inhibitory activity was present on the chromatographs, whereas some promotory activity was detected in some R_F sections. The first R_F section (R_F 0.0 to 0.07), which included the origin plus the application band, was promotory to hypocotyl growth. The next two R_F sections (numbered 2 and 3) from chromatographs of H3 extract, but not H1 extract, were also promotory.

The R_F of authentic ABA, which was determined by the assay of chromatographs loaded with authentic ABA on lettuce hypocotyls, was R_F 0.3 to 0.6. Therefore, most of the inhibitory activity of the inhibitor β fraction of apical extracts was not associated with phenolic-like compounds, but with a compound or compounds which had an R_F similar to that of authentic ABA.

The inhibitory zone present on chromatographs was further analysed by U.V. spectroscopy, TLC and gas chromatography. The zone was excised from the paper chromatographs and eluted in MeOH. An U.V. spectrum of the eluted fraction was obtained (figure 87) and compared with the U.V. spectrum of a methanolic solution of mixed isomers of synthetic ABA. The synthetic ABA was eluted previously from chromatographs. Both spectra were similar. The scan for ABA showed a maximum absorption peak at 235 nm, whereas the maximum for the eluted fraction was 240 nm. A second (shoulder) peak occurred at 280 nm on spectra of the eluted fraction and this may be attributed to an impurity. It is interesting to note that for both spectra $\lambda_{max.}$ was the same in alkali and acidic MeOH, and is at variance with other values given in the literature; Jenkins and Shepherd (1972) and Milborrow (1967) reported $\lambda_{max.}$ at 262 nm for an acidic ethanolic solution and 244 nm for the alkaline solution, Hall (1978) found $\lambda_{max.}$ to be 248 nm for both aqueous alkali and acidic solutions, and Ohkuma, Lyon, Addicott and Smith (1963) reported $\lambda_{max.}$ was 252 nm. The $\lambda_{max.}$ for the eluted fraction has been reported at 252 nm by Jenkins and Shepherd (1972) and 262 nm by Ramsay and Martin (1970b). In this study, the observed $\lambda_{max.}$ agreed with that given for alkaline conditions by Harbourne (1973).

The spectra tentatively suggested that ABA was present in the eluted fraction.

Replicates of the eluted fraction were also subjected to TLC and gas chromatography. The zone corresponding to the R_F of authentic ABA was eluted and methylated before gas chromatography. A peak corresponding to the same retention time as authentic ABA was found (figure 88) in both extracts. Identification was confirmed by co-chromatography with authentic ABA, but quantification between H1 and H3 extracts was not attempted.

The results of the analysis of the inhibitor β fraction suggested that ABA was the main inhibitor responsible for the inhibition of extension growth observed in wheat coleoptile sections and lettuce hypocotyls grown on eluates of the fraction.

3.2.4 Inhibitor β Levels and the Photoinduction of Bud Dormancy in *Alnus viridis*

A change in photoperiod from 16 hours (LD's) to 8 hours (SD's) had a marked effect on *Alnus* seedlings (figure 89). Short days inhibited extension growth and after 12 days of SD treatment, extension growth had ceased. The production of new leaves by apices also ceased and the formation of red bud scales was noted. By day 15, all buds had red scales and were considered to be dormant.

Mature leaves and apices were harvested separately on three occasions; day 0 (designated H1), day 7 (H2) and day 15 (H3) of SD treatment. Mature leaves and apices were also harvested from a single plant on day 21. The acidic ether-soluble extracts were prepared, chromatographed and the inhibitor β content determined by assay on wheat coleoptile sections and lettuce hypocotyls (details in section 2.3.1).

Inspection of chromatographs under visible and U.V. light revealed several bands on the developed chromatographs. The appearance of chromatographs was similar to chromatographs of *Alnus glutinosa* extracts illustrated in figure 17. The intensity of the light and U.V. reaction varied between chromatographs of leaf extract and chromatographs of apical extract, but the number and pattern of light and U.V. reacting bands was similar for both extracts.

3.2.4.1 Leaf extracts

Extract equivalent to 0.2 g DW of leaf material was chromatographed and assayed. The results of the assay on coleoptile

sections are presented as histograms in figures 90, 91 and 92, respectively for H1, H2 and H3. There was little or no growth activity, relative to that of the controls, on the first half (R_F 0.0 to o.5) of the chromatographs. No R_F sections were promotory to coleoptile growth whereas a zone of inhibition corresponding to the inhibitor β zone of Bennet-Clark and Kefford (1953) was found on all chromatographs. Within this zone, inhibition was most intense at R_F 0.5 to 0.7 and no differences in the intensity of inhibition was found between chromatographs of serially harvested extracts. The maximum inhibition detected by the assay of R_F sections was comparable to 10^{-5}M ABA.

The inhibitory activity of the β zone was assessed by measuring the area of inhibition. A nested ANOVA indicated there were no significant differences between the areas calculated for replicate extracts or between extracts from different harvest dates (Table 3.22).

Table 3.22 Nested ANOVA of coleoptile assay of leaf extracts (LD/SD expt.) involving three harvest dates (level 2), three extracts per harvest (level 1), and three chromatographs per extract (level 0). Data (areas of inhibition of β zones) are shown in figures 90, 91 and 92.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	2	690.82	345.41	0.6845 n.s.
Amongst extracts within dates	6	3 027.88	504.65	0.7354 n.s.
Extracts (Error)	18	12 352.10	686.23	
Total	26	16 070.8		

The data from the wheat assay, therefore, indicated that the inhibitory activity present in the inhibitor β fraction of LD, compared to SD treated leaves was not significantly different. The results of the hypocotyl assay of H1, H2 and H3 leaf extracts are shown in figures 93, 94 and 95, respectively and confirmed the results of the coleoptile assay. A major zone of inhibition corresponding to the inhibitor zone was located at R_F 0.5 to 0.8. The inhibitory activity of this zone was not significantly different between chromatographs of LD and SD extracts (Table 3.23) when the respective areas of inhibtion were compared.

Table 3.23 Nested ANOVA of hypocotyl assay of leaf extracts (LD/SD expt.) involving three harvest dates (level 2), three extracts per harvest (level 1), and three chromatographs per extract (level 0). Data (areas of inhibition of zones) are shown in figures 93, 94 and 95.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Amongst dates	2	3.156	1.578	0.0059 n.s.
Amongst extracts within dates	6	1 608.76	268.1	0.7334 n.s.
Extracts (Error)	18	6 580.47	365.58	
Total	26	8 192.39		

A second, less intense zone of inhibition was located in the first half of the chromatograph at R_F 0.2 to 0.4. The inhibitory activity of this zone was not detected on all chromatographs, but was greatest on chromatographs of two H1 extracts. The first R_F section of all chromatographs was promotory to hypocotyl growth.

Serial dilutions, equivalent to 0.025, 0.125, 0.25 and 0.500 g DW of leaf material were made of two extracts; one from H1 and one from H3. The results of the wheat assay (figures 96 and 97 for H1 and H3, respectively) and the lettuce assay (figures 98 and 99 for H1 and H3, respectively) were similar. At the lowest dilution, little or no growth activity was detected on chromatographs by either coleoptiles or hypocotyls. At the higher dilutions, on all chromatographs, the inhibitor β zone was the only zone of inhibition detected by coleoptile sections, whereas a second zone of inhibition was detected at R_F 0.2 to 0.4 by hypocotyls. However, this zone was not detected on chromatographs at lower dilutions. An increase in the amount of extract applied to chromatographs resulted in an increase in the area of inhibition within the β zone, and this increase was similar to that found with authentic ABA. At all dilutions assayed, the area of inhibition of the β zone was similar for chromatographs of LD extracts (H1) and SD extracts (H1 and H2).

The histogram data, obtained by bioassay, suggested that in leaves there was no change in the inhibitory activity of the β fraction during the photoperiodic induction of bud dormancy.

3.2.4.2 Apical extracts

All extracts were chromatographed and bioassayed at a dilution equivalent to 0.2 g DW of apical material. Inhibitory activity within the β zone was detected on all chromatographs assayed on coleoptile sections (figures 100, 101 and 102; H1, H2 and H3, respectively) and on lettuce hypocotyls (figures 103, 104 and 105 for H1, H2 and H3, respectively). Most R_F sections, with the exception of the first R_F section, which was promotory to hypocotyl growth, were inhibitory to coleoptile and hypocotyl growth. The inhibitor β zone was the main zone of growth inhibitory activity detected by the two bioassays. Within the β zone, inhibtion was most intense at R_F 0.5 to 0.7 and was comparable to 10^{-5}M ABA.

The area of inhibition within the β zone varied between chromatographs of extracts of the same and different harvest dates. An analysis of variance on the data obtained from the wheat (Table 3.24) and lettuce (Table 3.25) assays, indicated there were no significant differences between the area of inhibition within the β zone of chromatographs of serially harvested extracts.

Table 3.24 One-way analysis of variance of coleoptile assay of apical extracts (LD/SD expt.) involving three harvest dates (treatments) and three extracts per harvest (replicates). Data (areas of inhibition of β zones) are shown in figures 100, 101 and 102.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Treatments	2	1 310.76	655.38	1.4183 n.s.
Error	6	2 772.54	462.09	
Total	8	4 083.3		

Table 3.25 One-way analysis of variance of hypocotyl assay of apical extracts (LD/SD expt.) involving three harvest dates (treatments) and three extracts per harvest (replicates). Data (areas of inhibition of β zones) are shown in figures 103, 104 and 105.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Treatments	2	2 895.78	1 447.89	0.7878 n.s.
Error	6	11 027.34	1 837.89	
Total	8	13 923.12		

Serial dilutions of extracts were not possible because of the small quantity of apical material harvested (the max. quantity at any one harvest was 1.2 g DW). However, the quantity of extract remaining after the required number of aliquots, equivalent to 0.2 g DW of apical material, had been taken for assay, was collected and bulked for each of the three harvest dates. The combined extracts were assayed on lettuce hypocotyls at a dilution equivalent to 0.14 g DW of apical material. The results indicated that the inhibitory activity of the three different extracts was approximately the same (figure 106).

Leaves and apices were harvested from a single plant after 21 days of SD treatment. From the leaf material extracted, it was possible to assay six aliquots, equivalent to 0.25 g DW of leaf material. The results of the assay, three aliquots on coleoptiles and three on hypocotyls, are shown in figure 107. It was not possible to assay the apical extract on both assay systems because of insufficient material. The complete extract, equivalent to 0.19 g DW of apical material, was assayed on lettuce hypocotyls and the resultant histogram is shown in figure 107. The inhibitory activity, present on chromatographs of these extracts, was similar to previous extracts, including H1 extracts, and no increase in the inhibitory activity of the fraction of the leaf or the apical extract was detected.

The results of the determination of the inhibitor β content of leaves and apices suggested that there was no change in the levels during the photoinduction of dormancy. More inhibitor β activity was detected in apices than in leaves.

3.3 STUDIES ON INDUCTION OF BUD DORMANCY

The experimental approach designed to test the ability of exogenously applied hormones to induce dormancy in non-dormant plants can provide critical evidence on the role of hormones in the regulation of bud dormancy. Similarly, the induction of dormancy by experimental treatments, which alter the endogenous hormone balance, can provide supportive evidence for a hormonal mechanism.

3.3.1 Whole Plants

Seedlings of *Alnus viridis* were grown under conditions outlined in Chapter 2 (Materials and Methods). Seedlings were actively growing and under no obvious stress prior to treatments. Nitrogen fixing nodules were present on the root systems.

3.3.1.1 Root pruning

The height of seedlings with an intact or pruned root system was measured at regular intervals and the data expressed as percentage increase in height (figure 108). Pruning roots did not induce bud dormancy but seedling growth was affected by the pruning treatment. The experiment was repeated three times. The results of the first two attempts (figure 108a,b) indicated that increase in plant height was less immediately following pruning, but all plants quickly recovered to their original growth rate. On the third attempt, the proportion of root system excised was increased to the point where visible wilting was induced in the shoot system of some plants. Stem extension growth of these plants was severely affected and plants were slower to recover (figure 108c).

3.3.1.2 Application of growth regulatory substances

The ability of aqueous solutions of abscisic acid, CCC (an inhibitor of gibberellin biosynthesis), n-valeric acid, nonanoic acid, capric acid and a 1:1 solution of ABA + AMO 1618, to induce bud dormancy in seedlings maintained under conditions favouring growth, was tested. The height of seedlings, before and during the application treatments, was measured at regular intervals over a period of approx. 6 weeks (figures 109, 110 and 111). For control plants, the increase in height was approx. linear with time.

Application of 10^{-4}M abscisic acid did not induce bud dormancy in any of the plants. All apices continued to produce new leaves, and no red bud scales (a characteristic of dormant buds of *Alnus* species) were formed. Stem elongation was either unaffected (figures 109 and 111) or slightly suppressed (figure 110), but it never ceased. No leaf senescence or abscission occurred irrespective of whether the leaf had been partially immersed in ABA solution or sprayed. There were no marked differences between ABA-treated plants and control plants.

The treatment of plants with 10^{-3}M aqueous solutions of the growth retardant CCC, did not result in the formation of dormant buds. Extension growth was not markedly affected by the treatments (figures 109-111), although overall, the percentage increase in height of treated plants was less than control plants in some cases.

The extension growth of seedlings treated with a 1:1 aqueous mixture of 10^{-4}M ABA and 10^{-3}M CCC was only slightly suppressed (figures 109-111). Extension growth did not cease and no dormant buds were formed. Leaf abscission did not occur on any seedlings.

The application of short chain fatty acids; C5, C9 and C10, did not induce the formation of dormant buds. The growth of the seedlings was not markedly affected and no suppression of extension growth was observed (figure 112). There was evidence suggestive of slight extension growth stimulation by C9 (figure 112c). The immersion of the leaves in aqueous solutions of C9 and C10 resulted in severe leaf damage. The leaves lost their green colour and were completely bleached within 24 hours following immersion. Leaves were damaged to the extent that fragments of tissue became suspended in the solution. The damage was irreversible and occurred only in that portion of the leaf submerged in the solution. Spraying the plants with test solutions resulted in minor damage only; small areas or spots of bleached tissue were observed on the leaves. The C5, C9 and C10 acids were not toxic to the apices as no damage was observed, even when these acids were applied directly on to the bud surface by paint brush.

3.3.2 Excised Shoots

Young shoots that were excised from actively growing seedlings maintained under controlled growth conditions, continued to grow when cut bases were immediately submerged in water (figure 113, Table 3.26). Each shoot had 10 buds and these were labelled basipetally. On seedlings, only the apical bud (position 1) was growing and the lateral buds (positions 2 to 10) were assumed to be under the effects of apical dominance or correlative inhibition.

Four weeks after excision, 80% of the shoots had added at least one new node (Table 3.26) and new leaves were being produced by all apices. Extension growth was much slower in excised shoots than in shoots left on the plants; the percentage increases in length (height)

Table 3.26 The effect of ABA and the presence and absence of leaves on growth of isolated shoots of *Alnus viridis*. Values are of 10 shoots, each with 10 buds.

Treatment	% Increase in length (cm ± S.E.)	Total no. of growing buds	No. of shoots with new nodes
H_2O + leaves (Control)	12.1 ± 3.2	14	8
H_2) - leaves	11.7 ± 2.0	31	7
ABA + leaves	10.4 ± 3.6	12	7
ABA - leaves	10.6 ± 2.8	16	6
Whole plant	36.2 ± 5.4		

were 12 and 36, respectively (Table 3.26). Leaf abscission occurred at some bud positions (figure 113b). Most of the leaves at basal positions of the excised shoot abscised, whereas leaves at apical positions remained on the stem. At basal positions where leaf abscission occurred, buds were stimulated to grow if abscission had taken place within 10 to 14 days following excision. No adventitious roots formed during the 4 week period.

The removal of all leaves from the shoots, at the time of excision, did not markedly affect the percentage increase in length or the number of shoots which continued to grow (Table 3.26). However, outgrowth of lateral buds was stimulated at some positions on most shoots (figure 113).

When the bases of excised shoots were placed in solutions of 10^{-4}M ABA, the growth of the apical bud was not affected (Table 3.26, figure 113). Shoots continued to increase in length by adding new nodes, although the percentage increase in length was slightly less for shoots with and without leaves (10.4 ± 3.6 and 10.6 ± 2.8, respectively) than the respective controls (12.1 ± 3.2 and 11.7 ± 2.0). Abscisic acid had little effect on the number of leaves retained at all positions on the stem when compared to the water control. For example, at bud positions 1, 2 and 3, the number of leaves retained on ABA treated shoots was 10, 9 and 4 respectively, whereas for the control shoots the numbers were 9, 8 and 6, respectively. Abscisic acid treatment did not promote leaf abscission.

Abscisic acid was able to arrest the outgrowth of lateral buds which had been stimulated to grow by the removal of the leaves. It was not determined whether or not dormancy had been induced in these buds, although it was observed that the formation of red bud scales, which is a characteristic feature of dormant buds, did not occur.

The results indicated abscisic acid was not able to induce dormancy in apical and lateral buds of excised, young growing shoots.

3.4 STUDIES ON THE MAINTENANCE OF BUD DORMANCY

It is possible that the hormonal mechanisms involved in the induction and maintenance of bud dormancy are different. Abscisic acid and other endogenous growth inhibitors might have a role in maintenance of bud dormancy but not in the induction phase of dormancy. The ability of ABA to prolong the dormancy of buds after their transfer to an environment favouring growth was tested in three species; *Alnus*, *Populus* and *Salix*. The ability of four short chain fatty acids (C5, C8, C9 and C10) and the plant growth retardant CCC, to prolong dormancy, were also tested.

The experimental system (given in detail in section 2.9.4) involved the excision of lengths of young shoots, complete with the apical bud and nine nodes, from trees which had entered dormancy under natural conditions. The shoots (10), with their cut bases submerged in test solutions, were then transferred to controlled conditions favouring growth (16 hour photoperiod and high temperatures 20^{o}C). The number of buds that broke dormancy and commenced growth after a period of incubation was recorded for each node or bud position. The number of growing buds was taken as an indication of the ability of the test solution to prolong dormancy.

The uptake of test solutions by the shoots was observed in all treatments. Initially, uptake was very rapid, especially in cases where the nodes were foliated. Generally, for each replicate set of 10 shoots, 50 ml of test solution was taken up within the first 10 hours.

3.4.1 Poplar (*Populus nigra* Italica)

Leafless shoots of *Populus* were harvested in early spring

(July). In the control treatment 62% of the buds had broken dormancy after 17 days (Table 3.27).

Table 3.27 The effect on bud burst of various growth regulator applications to the base of isolated shoots of *Populus nigra* Italica. The values represent total number or mean percentage bud burst per treatment. Each treatment comprised four replicates, each of 10 shoots, with 10 buds per shoot.

Treatment	Total no. growing buds	Mean % bud burst
H O (Control)	248	62.0
ABA 10^{-4}	206	51.5 *
ABA 10^{-5}	289	72.3
C5	256	64.0
C8	209	52.3
C9	243	60.8
CCC	256	64.0
ABA + CCC	182	45.5 **
	$LSD_{0.01}$ = 14.1	$LSD_{0.05}$ = 10.5

Not all the buds on a shoot showed the same tendency to break dormancy (figure 114). The position of the bud on the stem was a significant factor in determining whether that bud would grow (Table 3.28). Basal buds at positions 7 to 10 consistently showed a growth response, whereas apical buds, especially at positions 2 and 3, remained inactive. The buds at position 2 on stems were markedly smaller than the other buds. The buds at intermediate positions (4, 5 and 6) showed a progressive increase in the tendency to commence growth.

The total number of growing buds was significantly less when shoots were treated with 10^{-4}M ABA, either alone (51.5%, $p < 0.05$) or in combination with CCC (45.5%, $p < 0.01$) (Table 3.27). The inhibitory effect of ABA on bud burst was not consistent at all positions on the stem. There was a significant interaction ($p < 0.001$) between ABA and the position of the bud (Table 3.28). Bud burst on the lower half of the shoot was significantly less in the presence of ABA than in its absence, whereas at the apex, bud burst was significantly higher than the control (figure 114). Bud burst at intermediate positions (3 to 7)

Table 3.28 Two-way ANOVA of bud burst in *Populus nigra* Italica involving 10 bud positions on the stem (factor A) and eight treatments (test solutions, factor B). Data are shown in figures 114, 115 and 116.

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Subgroups	79	1908.25	24.155	11.6002 ***
A (columns, bud position)	9	1021.97	113.55	54.5313 ***
B (rows, treatments)	7	208.68	29.811	14.3164 ***
A × B Interaction	63	677.60	10.7556	5.1653 ***
Total subgroup (within error)	240	499.75	2.082	
Total	320			

was not affected. The promotory response elicited by 10^{-4}M ABA at the apex did not occur when in combination with CCC (figure 115). However, at other bud positions, the effect of the combined solution was similar to that of ABA when applied alone. Abscisic acid at 10^{-5}M did not have a significant effect on total bud burst, although the mean percentage of buds which had commenced growth (73%) was higher in the presence of 10^{-5}M ABA than in its absence (62%). When the apical buds (position 1) were considered alone, the presence of 10^{-5}M ABA caused a significant ($p < 0.05$) increase in bud burst (figure 114). The response of other buds was similar to that found in the absence of ABA.

Overall, the buds showed no significant responses to any of the three short chain fatty acids tested; C5, C8 and C9, although in the presence of C8, 52.3 per cent of the buds had commenced growth after 17 days, whereas in the water control the mean percentage was 62 (Table 3.27). When buds at positions 1 and 4 were analysed separately, the response to C8 was found to be significant ($p < 0.05$) (figure 116). At both positions, C8 significantly depressed bud burst. Similarly, buds at position 3, in the presence of C9, showed a significant ($p < 0.05$) increase in bud burst, although over the whole shoot C9 had no significant effect on bud burst (Table 3.27). A comparison of bud burst at positions 3 and 4, in the presence of C8 and C9, showed that C9 promoted bud burst relative to C8 (figure 116). There were no

significant differences between the mean percentage bud burst in the presence or absence of C5.

In the presence of 10^{-4}M CCC, the overall response of buds was not significantly different ($p > 0.05$) to the control (Table 3.27). The interaction between the ability to grow, and bud position, was not altered (figure 115).

The mean percentage of buds that had commenced growth was significantly less ($p < 0.01$) when CCC in combination with ABA was applied (Table 3.27). However, the ability of basal buds (positions 8 to 10) only was significantly affected and the inhibitory response at each of these basal positions was not significantly different ($p < 0.05$) from the response elicited by 10^{-4}M ABA alone (figure 115). This suggested the inhibitory response was due to the presence of ABA and not CCC.

Although no detailed data were recorded, the formation of adventitious roots was not readily apparent on any of the shoots.

3.4.2 Alder (*Alnus viridis*)

The ability of test solutions of ABA, CCC and three short chain fatty acids to prolong the dormancy of green alder (*Alnus viridis*) buds was tested on shoots harvested in autumn/winter (April) and late winter (July). The two harvests were made in different years but from the same trees.

3.4.2.1 Late winter harvest

At this harvest all shoots had lost their leaves. The percentage of buds that had commenced growth after 40 days was 29% for shoots incubated in water alone (Table 3.29). Buds at different positions on the stem exhibited tendencies to remain inactive and the low percentage bud burst reflected the inability of the apex, and basal buds at positions 7 to 10, to commence growth (figure 117). The varied responses of buds at different positions on the stem were significantly different ($p < 0.001$, Table 3.30), indicating that the position of the bud on the stem was an important factor as to whether or not that bud would commence growth. The tendency to commence growth was highest for buds at positions 2, 3 and 4 on the stems, and lowest for buds at the apex where no buds had commenced growth. Buds at intermediate and basal positions showed a progressive decline in the ability to grow.

Table 3.29 The effect on bud burst of the presence and absence of leaves and of various growth regulator applications to the bases of isolated shoots of *Alnus viridis*. The values represent total number or mean percentage bud burst at the late winter and autumn harvest dates with the leaves removed at the beginning of the experiment. No leaves were present at the late winter harvest date. Each treatment comprised 10 shoots, each with 10 buds, and was replicated four times (late winter harvest) and three times (autumn harvest).

	LATE WINTER		AUTUMN			
			+ Leaves		(-) Leaves	
Treatment	Total no. growing buds	Mean %	Total no.	%	Total no.	%
H_2O	117	29.0**	22	9.2	18	7.5*
ABA 10^{-4}M	22	5.5**	26	10.8	12	5.0*
ABA 10^{-5}M	83	20.8**	-	-	-	-
C5	83	20.8**	-	-	-	-
C8	50	12.5**	-	-	-	-
C9	24	6.0**	-	-	-	-
CCC	81	20.3**	18	7.5	5	2.1%
ABA + CCC	9	2.3**	15	6.3	5	2.1%
Mean		14.2				4.2
	$LSD_{0.01}$ = 6.88 $LSD_{0.05}$ = 5.04				$LSD_{0.05}$ = 4.3	

Table 3.30 Two-way ANOVA of bud burst in *Alnus viridis* (late winter harvest date) involving 10 bud positions on the stem (factor A) and eight treatments (test solutions, factor B). Data shown in figures 117, 118 and 119.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Subgroups	79	1 095.37	13.8654	12.4062 ***
A (columns, bud position)	9	613.59	68.1767	61.0014 ***
B (rows, treatments)	7	290.80	41.543	37.1707 ***
A × B Interaction	63	190.98	3.0314	2.7124 ***
Within subgroup error	240	268.23	1.1176	
Total	320			

When bud burst was assessed in the presence of each of the test solutions, a significant ($p < 0.01$) decrease in the mean number of buds that had commenced growth was found (Table 3.29).

Abscisic acid at 10^{-5}M and 10^{-4}M caused a significant decrease in the mean number of buds bursting, from 29% to 20.8% ($p < 0.05$) and 5.5% ($p<0.01$) respectively (Table 3.29). In the presence of ABA at both concentrations, bud burst was affected at all positions on the stem where buds had commenced growth in the absence of ABA (figure 117). At the higher concentration, 10^{-4}M, bud burst was significantly depressed ($p < 0.01$) at most positions, especially positions 2 to 6 and 9 and 10, whereas at the lower concentration, 10^{-5}M, bud burst was significantly less ($p < 0.01$) at positions 3, 4, 5 and 6 only. Bud burst was least affected at position 2 on the stem.

The mean percentage bud burst (20.3) was significantly less ($p < 0.05$) in the presence of 10^{-3}M CCC than in its absence (29%) (Table 3.29). The reduction in bud burst was significant at intermediate positions (3-6) on the stem only (figure 118). At other positions, bud burst was not affected, although at position 10, all buds had remained inactive.

The combined 1:1 solution of ABA and CCC was most effective in depressing bud burst (Table 3.29). Overall, the mean number of buds that had commenced growth was 2.3% in the presence of the combined solution compared with 29% in its absence. The difference was significant ($p < 0.01$). The ability of buds to grow was significantly ($p < 0.01$) depressed at all positions on the stem (figure 118) and total arrest of bud burst occurred at positions 4 and 6 through to 10. At position 2, the tendency to grow was highest but the number of growing buds was still significantly less than in the control. The mean percentage bud burst (2.3%) was lower than that for ABA (5.5%) and CCC (20.3%) alone. However, the difference between these values was significant ($p < 0.01$) in the case of CCC and ABA + CCC, only. Nevertheless, at several bud positions, for example positions 4, 6, 7 and 8, complete arrest of bud burst occurred in the presence of the combined solution but not in the presence of ABA alone. This evidence is suggestive of an interaction between ABA and CCC.

Each of the three short chain fatty acids tested; C5, C8 and C9, were effective in reducing bud burst (Table 3.29). The effectiveness

increased with the increase in the length of the carbon chain, with C9 being most effective and C5 the least. For all three short chain fatty acids, the mean percentage bud burst was significantly less ($p < 0.001$ for C9 and $p < 0.01$ for C5 and C8) than the percentage bud burst in water (Table 3.29). In the presence of C5, the mean percentage bud burst was 20.8%, whereas for C8 and C9 the mean percentage was 12.5% and 6.0%, respectively.

Pentanoic acid (C5) significantly reduced ($p < 0.01$) bud burst at positions 3, 4 and 5 on the stem, but had no effect on bud burst at other positions (figure 119). The response of buds to C9 was similar to their response to 10^{-4}M ABA. Nonanoic acid (C9) arrested all bud burst at positions 6 to 10 and significantly reduced ($p < 0.01$) bud burst at positions 2 to 5 (figure 119). In the presence of C8, the mean number of buds growing at most positions on the stem was intermediate between the values for C5 and C9 (figure 119). Bud burst at positions 3 to 6 was significantly less ($p < 0.01$) than that in controls. Bud burst at basal positions (7-10) was lower than that observed in the presence of C5 but was not completely arrested as was the case for C9. With all three short chain fatty acids, the response of buds at position 2 on the stem was least affected and only C9 significantly reduced bud burst at this position.

3.4.2.2 Winter and autumn harvests

At these harvest dates (April), leaves were still present on the shoots and in addition to the effect of solutions of ABA, CCC and a mixture of ABA and CCC on bud burst, the effect of the presence or absence of leaves on bud burst was also tested. Initially, the winter harvest experiment had been conducted in the year (1977) following the late winter harvest (1976). Treatments including the short chain fatty acids and the presence and absence of leaves were set up. However, after 60 days of incubation only 15 buds, totalled over all treatments, had commenced growth. Therefore the results for this experiment were not analysed and are not presented. The experiment was repeated in 1979 but shoots were harvested three weeks earlier (designated autumn harvest)."

For an initial period of 24 to 28 hours, the shoots with leaves attached showed greater uptake of liquid from incubation vessels than leafless shoots. Within 10 days all the leaves had abscised.

Adventitious root formation was apparent on a few shoots (<10) from both the autumn and late winter harvest. Usually, a single root was seen on a shoot and the shoots with adventitious roots were randomly distributed within the treatments.

Over all comparable treatments (- leaves, water, ABA, CCC and ABA + CCC) there was a marked but non-significant decrease ($F = 2.3595$) in the mean percentage bud burst at the autumn harvest compared with the late winter harvest (Table 3.29). At the late winter harvest, the mean percentage of buds which had commenced growth after 40 days was 14.2% compared with 4.2% at the autumn harvest.

Bud burst on shoots (figure 120) was generally similar to that observed on shoots from the late winter harvest. However, at the autumn harvest some apical buds showed a growth response, whereas no apical buds had commenced growth on shoots at the late winter harvest. At both harvests, basal buds showed a tendency to remain inactive. Therefore, as had been found for shoots of the late winter harvest, the growth response of buds at different positions on the stem differed markedly (figure 120).

The mean percentage bud burst did not vary with the treatment factor (different test solutions) but there was a significant difference ($p < 0.001$) due to the leaf factor (presence or absence) (Table 3.31).

Table 3.31 Two-way ANOVA of bud burst in *Alnus viridis* (autumn harvest date) involving the presence or absence of leaves (factor A) and four treatments (test solutions, factor B). Data are shown in figure 120.

Source of variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Subgroups	7	132.26	18.89	2.835 n.s.
A (columns, (+) or (-) leaves)	1	70.04	70.04	9.7685 ***
B (rows, treatments)	3	52.13	17.38	2.2420 n.s.
A × B interaction	3	10.09	3.36	0.4686 n.s.
Within subgroup error	16	114.7	7.17	
Total	23			

However, there was no evidence for an interaction between the leaf factor and the treatment factor. The analysis shown in Table 31 (two factor ANOVA) also suggested that, in the presence of treatment factors (ABA, CCC and ABA + CCC and H_2O), the reduction in mean percentage bud burst may not be real. Therefore, the data obtained in the presence and absence of leaves was analysed separately.

When leaves were present, there were no significant differences between the mean percentage bud burst found for the various treatments (Table 3.29, Fs = 0.6410). A very low percentage of buds (9%) had commenced growth after 40 days incubation in water. The growth retardant, CCC, either alone or in combination with ABA, reduced the mean percentage bud burst to 7.5% and 6.3%, respectively. However, the decrease in bud burst was not significant ($p > 0.05$). In the presence of ABA, the mean percentage bud burst was 10.8%, but this was not significantly higher than bud burst in its absence (9.2%). In the presence of leaves, only buds at apical positions showed a tendency to grow and the low mean percentage bud burst figures, reported above, were a reflection of this. The apex consistently showed a growth response, whereas buds at basal positions 5-10 showed no growth response. The buds at positions 2, 3 and 4 on the stem showed a progressive decrease in the ability to commence growth. Differences in the ability of buds to grow at different positions on the stem were significant ($p < 0.001$) (Table 3.32).

Table 3.32 Two-way ANOVA of bud burst in *Alnus viridis* (autumn harvest date) involving eight bud positions on the stem (factor A) and eight treatments (test solutions, factor B). Data are shown in figure 120.

Source of Variation	Degrees of freedom	Sum-of-squares	Mean-squares	Fs
Subgroups	63	214.07	3.3979	5.6728 ***
A (columns, bud position)	7	159.12	22.7314	37.9500 ***
B (rows, treatments)	7	16.54	2.3629	3.9448 ***
A × B interaction	49	38.41	0.7839	1.3087 n.s.
Within subgroup error	128	76.67	0.599	
Total	191			

In the absence of leaves, all three test solutions caused a marked reduction in the mean percentage bud burst when compared with the water control (Table 3.29). When shoots were incubated in water, the presence or absence of leaves had no significant effect on bud burst. The mean percentage bud burst in the presence of leaves was 9.2% and 7.5% in the absence of leaves. Abscisic acid in the absence of leaves caused a reduction in the mean number of buds which had commenced growth, but this effect was not significant ($p < 0.05$) when all the buds on the shoot were considered (Table 3.29). However, when buds at the apex are considered separately, a significant reduction ($p < 0.05$) in bud burst did occur (figure 120).

Solutions of CCC were effective in significantly reducing ($p < 0.05$) the mean percentage bud burst in the absence of leaves from 7.5% to 2.1% (Table 3.29). Bud burst at the apex was most affected by the presence of CCC, and the reduction in bud burst at this stem position was also significantly different ($p < 0.001$) from the response elicited by ABA (figure 120). A 1:1 mixture of CCC and ABA also caused a significant decrease ($p < 0.01$) in the number of buds that had commenced growth at the apex. However, at the apex, the growth response was not significantly different ($p > 0.05$) from the response elicited by CCC alone. Similarly, when buds at all positions on the stem were considered, the mean percentage bud burst was not significantly different from the CCC treatment (Table 3.29). In both cases, CCC and ABA + CCC, the mean percentage bud burst was 2.1%. The evidence suggested that the response of the combined solution was mostly due to CCC.

3.4.3 Willow (*Salix alba/babylonica*)

The experimental design in this study was altered so that the bud position and the leaf effect (presence or absence) could be studied in more detail. The treatments, which involved various degrees of defoliation, lengths of shoots and test solutions are outlined in detail in section 2.9.4. Shoots were harvested in April. To balance the limited number of suitable shoots against the replication necessary for each treatment, the short chain fatty acids were not included in the treatments. Despite this, only two replicates of each treatment or experimental unit (which comprised 10 shoots) were possible. It was decided that replication could be best achieved by repeating the whole

experiment the following year, and pooling the results. However, in the first year during the incubation period, several shoots in each replicate of ten, developed wilt symptoms, which were followed by the appearance of black lesions on the stem and bud surfaces, and eventually died. Although for the remaining healthy shoots, bud burst was scored after 24 days, the experiment was abandoned and the results excluded from the analysis of the following year's results. The results, which are presented below, represent percentage bud burst based on only two replicates (albeit comprising 10 shoots each) and therefore no statistical separation of the major differences was attempted.

After 1 week of incubation, all the leaves had abscised from all treatments in those experiments where the leaves had not been removed at the beginning of the experiment. After 4 weeks of incubation, all shoots consistently showed the formation of adventitious roots on the submerged shoot bases, irrespective of the treatment. However, root formation was observed to be less in shoots treated with ABA solutions, and in leafless shoots. Approximately 20 roots per stem were observed on shoots incubated in water.

The number of buds that had commenced growth after 4 weeks of treatment was always less than 12.5% and was affected by the presence or absence of leaves (Table 3.33).

Table 3.33 The effect on bud burst of the presence and absence of leaves and of various growth regulator applications to the bases of isolated shoots of *Salix alba/babylonica*. The values represent mean percentage bud burst ± standard errors with the leaves removed from all nodes [(-) leaves] or from the five apical nodes (2/L) or from the five basal nodes (L/2) at the start of the experiment. Each treatment comprised 10 shoots, each with 10 buds, and was replicated twice.

Treatment	LEAF FACTOR					
	(+) Leaves	(-) Leaves$_1$	L/2$_2$	2/L$_3$	$\bar{Y}_{123}$	$\bar{\bar{Y}}$
H_2O	12.5 ± 1.5	4.5 ± 0.5	12.0 ± 2.0	9.5 ± 0.5	8.7	9.6
ABA 10^{-4}M	6.5 ± 4.5	0.0	3.5 ± 0.5	1.0 ± 1.0	1.5	2.8
ABA 10^{-5}M	10.0 ± 4.0	0.5 ± 0.5	4.0 ± 2.0	4.5 ± 3.5	3.0	4.8
CCC	4.0 ± 3.0	3.5 ± 1.5	7.0 ± 3.0	3.0 ± 1.0	4.5	4.4
ABA + CCC	2.0 ± 2.0	0.5 ± 0.5	0.0	0.0	0.2	0.6
Mean	7.0	1.8	5.7	3.6		

In the presence of leaves, over all test solution treatments the mean percentage bud burst was 7% compared with 1.8% in the absence of leaves. When the leaves were removed from the apical half of the shoot (leaf treatment factor designated 2/L), the mean percentage bud burst was 3.6%, whereas the removal of the leaves from the basal half of the shoot (leaf treatment factor designated L/2) resulted in 5.7% bud burst.

Solutions of ABA, CCC and a 1:1 mixture of ABA and CCC were all effective in reducing the number of buds that had commenced growth (Table 3.33). Over all the leaf treatments, the mean percentage bud burst in the presence of water was 9.6% whereas in the presence of 10^{-4}M ABA, 10^{-5}M ABA, CCC and the combined solution of ABA + CCC, the mean percentage bud burst was 2.8, 4.8, 4.4 and 0.6, respectively.

For the control treatments, the mean percentage bud burst was 12.5% when leaves were present and 4.5% when leaves were absent. Irrespective of the presence or absence of leaves, a marked reduction in bud burst occurred in the presence of 10^{-4}M ABA. However, the mean percentage bud burst in the presence of leaves (6.5%) had a large standard error (±4.5) associated with it, and therefore, the 10^{-4}M ABA effect in the presence of leaves may not be real. Abscisic acid at 10^{-5}M, only slightly reduced the percentage (from 12.5% to 10.0%) bud burst in the presence of leaves whereas when the leaves were removed, 0.5% of the buds grew compared with 4.5% in the controls. In the absence of leaves, the differences between the control treatment and 10^{-5}M ABA was large compared with the difference in the presence of leaves (Table 3.33), and suggests there was an interaction between the response of buds to 10^{-5}M ABA and the presence and absence of leaves.

Solutions of CCC also caused a reduction to 4.0% in bud burst in the presence of leaves but only a slight reduction, 4.5% to 3.5%, in the absence of leaves (Table 3.33). When leaves were present, solutions of a 1:1 mixture of ABA and CCC were more effective in depressing bud burst than solutions of ABA or CCC alone.

When the position of the bud on the stem was considered, the low percentage of buds that had commenced growth in the presence of leaves was reflective of the growth response of mainly apical buds (figure 121a). Most basal buds remained inactive although a few had commenced growth in some treatments. For shoots incubated in water,

the buds at the apex had the greatest tendency to grow, followed by buds at positions 2 to 5. Buds at these positions showed a progressive basipetal decline in bud burst. The treatment of shoots with test solutions resulted, mainly, in a decrease in bud burst at the apical positions (1-4) (figure 121). However, ABA at 10^{-5}M caused basal buds at positions 5 to 9 to commence growth. The 1:1 ABA + CCC solution was effective in arresting bud burst at the apical positions.

In the absence of leaves, bud burst at the apical positions was also depressed, both in the presence and absence of test solutions (figure 121b). Abscisic acid, at both concentrations, completely arrested bud burst at all positions on the stem whereas CCC had little effect on bud burst at the apex.

The removal of the leaves from the basal half of the shoots only (treatment L/2), did not markedly affect the number of buds that had commenced growth when shoots had been incubated in water (figure 122b). However, there was a reduction in the mean percentage bud burst from 12.5% to 9.5% (Table 3.33) when leaves were removed from the apical half of the shoot (treatment 2/L). For both treatments, L/2 and 2/L, bud burst was markedly less than when all the leaves were present on the shoot but greater than bud burst when all the leaves were absent (Table 3.33).

The number of buds that had commenced growth in the presence of 10^{-4}M or 10^{-3}M CCC was higher for treatment L/2 than treatment 2/L. Abscisic acid treatment resulted in 3.5% and 1.0% bud burst for L/2 and 2/L, respectively. Curiously, the CCC value for L/2 was markedly higher than when all the leaves were present (Table 3.33).

For shoots incubated in solutions of 10^{-5}M ABA, bud burst was the same for L/2 and 2/L treatments, 4.0% and 4.5% respectively, but considerably less than bud burst (10%) when all the leaves were present.

In the presence of solutions of a 1:1 mixture of ABA and CCC, bud burst was totally depressed for both 2/L and L/2 treatments, whereas a few buds (0.5%) had commenced growth when all the leaves were absent (Table 3.33).

When shoots were incubated in water, bud burst at each corresponding position on the stem was similar for the L/2 and 2/L treatments (figure 122) and similar to that when all the leaves were

present (figure 121). Mostly apical buds had commenced growth, and the depression of total bud burst in the presence of each test solution was reflective of the depression of bud burst at apical positions. In the presence of test solutions, bud burst at the apical positions was less for the 2/L treatment (figure 122a) than the L/2 treatment (figure 122b), except for the 10^{-4}M ABA and ABA + CCC treatments where all bud burst was arrested at all apical positions. When leaves were absent from the basal half of the shoot, solutions of 10^{-5}M ABA and CCC slightly stimulated bud growth in these basal positions.

The data in figure 122 suggested the absence of leaves from either the apical or basal half of the shoot did not markedly affect bud burst at the apical positions on the stem. However, the removal of leaves from the apical half of the shoot had a greater inhibitory effect on bud burst than did removal of the basal leaves. In the case of apical buds, the presence of leaves at some other position on the stem was sufficient to counter the inhibitory influence due to the complete removal of the leaves, whereas in the added presence of ABA or CCC solutions the remaining leaves were ineffective in overcoming the inhibitory influence of ABA or CCC.

The isolation of the apical section of the shoot from the basal section of the shoot, by cutting the stem above node 6, had a marked effect on the growth response of basal buds (Table 3.34). The response of the buds on the isolated apical half of the shoot, in the presence or absence of leaves, was slightly less or comparable to the response of the apical buds on the intact or uncut shoots. Over all treatments, for the apical buds in the presence of leaves on intact shoots, the mean percentage bud burst was 5.5% and 3.8% on the halved or isolated shoots whereas in the absence of leaves, the mean percentage was 2.0 on the intact shoots and 1.1% on cut shoots.

When bud burst was considered at each position on the stem, a similarity between the apical buds on cut and uncut shoots was observed in the presence (figure 123a) or absence (figure 123b) of leaves over all treatments (figure 123). The buds that had commenced growth were at the most apical positions, with bud burst being highest at the apex followed by the other stem positions in basipetal sequence. Bud burst was depressed at these apical positions by the presence of test solutions.

Table 3.34 The effect on bud burst of the isolation of apical and basal sections of isolated shoots of *Salix alba/babylonica*, and the subsequent effect of growth regulators and the presence and absence of leaves. Values represent mean percentage bud burst ± standard error, with the isolation of apical and basal sections and the removal of leaves at the start of the experiment. Each treatment comprised 10 shoots, each with 10 buds, and was replicated twice.

	(+) Leaves		(-) Leaves	
	Isolated ("cut")	Intact	Isolated ("cut")	Intact
Apical Section				
H O	9.0 ± 1.5	12.0 ± 1.0	3.5 ± 0.5	4.5 ± 0.5
ABA	2.5 ± 1.0	5.5 ± 3.5	0.0	0.0
CCC	3.5 ± 1.5	3.0 ± 2.0	1.0 ± 1.0	3.0 ± 1.0
CCC + ABA	0.0	1.5 ± 1.5	0.0	0.5 ± 0.5
$\bar{Y}$	3.8	5.5	1.1	2.0
Basal Section				
H O	4.0 ± 1.0	1.0 ± 0.5	4.0 ± 1.0	0.5 ± 0.5
ABA	1.0 ± 1.0	1.0 ± 1.0	2.0 ± 1.0	0.0
CCC	2.0 ± 1.0	0.0	1.0 ± 1.0	0.5 ± 0.5
ABA + CCC	0.0	0.5 ± 0.5	0.0	0.0
$\bar{Y}$	1.8	0.6	1.8	0.3

Generally, the response of apical buds to the test solution was similar in intact and cut shoots when compared in the presence and absence of leaves. However, an exception to this was the response of apical buds to solutions of CCC in the absence of leaves. In this case, CCC was more effective in depressing bud burst at apical positions in cut shoots than in intact shoots.

Isolation of the basal section from the apical half of the shoot markedly affected the number of basal buds that had commenced growth. Over all treatments, the mean percentage bud burst for intact shoots was 0.6% in the presence of leaves and 0.3% in the absence of leaves whereas in the cut shoots, the mean percentage bud burst was 1.8% in the presence and absence of leaves (Table 3.34). The increase in the mean percentage bud burst was mostly due to increased bud burst on cut shoots incubated in water. Bud burst in the presence of leaves was 1.0%

for intact shoots and 4.0% for the cut shoots, whereas in the absence of leaves the bud burst was 0.5% and 4.0% for intact and cut shoots, respectively. The response of the basal buds to the test solutions ABA, CCC and ABA + CCC was similar in intact and cut shoots when compared in the presence and absence in leaves (Table 3.34).

The increased bud burst in isolated basal sections incubated in water, in the presence and absence of leaves, was the result of stimulation of bud burst at the uppermost position on the cut shoot, which corresponded to position 6 on intact shoots (figure 123). In the presence of leaves, the stimulation of bud burst at the new apex was arrested by the presence of solutions of ABA but not by solutions of CCC (figure 123a), whereas in the absence of leaves both ABA and CCC treatments arrested bud growth (figure 123b). However, bud burst at position 8 on the stem was slightly stimulated by 10^{-4}M ABA. The 1:1 mixture of ABA and CCC was totally effective in depressing bud burst at all basal positions, irrespective of the presence or absence of leaves (figure 123).

At the time of the second harvest, shoots from a second but apparently much younger tree of the same species, were also harvested and the ability of solutions of ABA and CCC, alone and in combination, and solutions of decanoic acid (C10) to prolong the dormancy of the buds, tested.

All test solutions were tested in the presence of leaves only, because the amount of shoot material that was obtainable was limited. Similarly, it was not possible to replicate each treatment more than twice.

As in previous experiments, all shoots showed rapid uptake of the test solutions in the first 24 hours. Leaf senescence and abscission occurred on all shoots in all treatments after 10 days. Shoots treated with C10 retained their leaves the longest, whereas the shoots treated with ABA, alone or in combination with CCC, were observed to lose their leaves 2 to 3 days earlier than the other treatments. At the submerged shoot bases, adventitious roots were observed on shoots in all treatments irrespective of the degree of bud burst. The longest roots were present on shoots incubated in the absence of test solutions, although shoots treated with C10 also showed comparable root formation and development. Abscisic acid was observed

to inhibit root formation and elongation.

The percentage of buds that had commenced growth after 4 weeks of incubation in water was 15.5% (Table 3.35). In the presence of solutions of 10^{-4}M ABA and CCC, the number of buds was reduced to 1.0% and 3.5%, respectively. Bud burst on shoots treated with C10 was stimulated and 27% of the buds had commenced growth. The combined presence of ABA and CCC was effective in depressing all bud burst. With the exception of the ABA treatment, the number of buds that commenced growth in the presence or absence of test solutions was similar to that found for the older tree. Abscisic acid was more effective in depressing bud burst in the younger tree than the older tree.

Table 3.35 The effect of growth regulators on bud burst in a young and mature tree of *Salix alba/babylonica*. Values represent mean percentage bud burst ± standard error with the leaves removed at the start of the experiment. Each treatment comprised 10 shoots, each with 10 buds, and was replicated twice.

Treatment	Percentage bud burst	
	Young tree	Mature tree (from Table 3.34)
H_2O	15.5 ± 1.0	12.5 ± 6.5
ABA	1.0 ± 1.0	6.5 ± 4.5
CCC	3.5 ± 2.5	4.0 ± 3.0
ABA/CCC	0.0	2.0 ± 0.0
C10	27.0 ± 1.0	-

The responses of buds at different positions on the stem was also similar to corresponding buds on shoots from the older tree. The apical buds (positions 1, 2 and 3 on the stem) showed the greatest tendency for growth, whereas basal buds remained inactive (figure 124). Bud burst was highest at the apex and showed a progressive decrease at positions 2 to 6. CCC markedly reduced bud burst at apical positions whereas ABA arrested all bud burst at these positions. Decanoic acid had very little effect on apical and intermediate buds but stimulated the basal buds especially at position 10.

The results of the study on willow suggested that bud position and the presence and absence of leaves were of major importance in determining whether the bud would commence growth. In willow, only apical buds were capable of growth on shoots harvested during the winter, but basal buds were capable of growth if they were isolated from the apical buds. The importance of bud position was also shown for poplar and alder species.

For all species, bud burst was affected by treatment with ABA and CCC but the response was complicated by the position of the bud on the stem, the presence and absence of leaves, and the time of year. The application of ABA and CCC together to shoots was most effective in delaying bud burst. Three short chain fatty acids C5, C8 and C9 inhibited bud burst in alder and poplar, but C10 was stimulatory to bud burst in willow.

3.5 STUDIES WITH ASEPTICALLY CULTURED BUDS AND SHOOTS

Plant tissue culture methods used for the rapid micropropagation of woody plants (Abbott, 1977) have potential use as tools in physiological studies. The tissue culture methods provide a more refined approach to studies of the hormonal control of growth processes. Growth responses of individual buds and shoots can be studied in relative isolation under controlled environment conditions. Similarly, the possibility exists for the establishment of a more refined bioassay system for the detection of specific growth regulatory substances. It was envisaged that the growth of aseptically cultured shoots could be manipulated by changing the composition of the media.

3.5.1 Suitability of Species

The ability of bud explants of one *Alnus* species and four *Populus* species to produce multiple shoots was tested. Single buds and/or shoots with three leaves were to be isolated from shoots 30 to 80 mm long.

Initial attempts concentrated only on *Alnus viridis* using a method known to be highly successful for the rapid micropropagation of *Populus* species *in vitro*. However, *Populus* species were found to be more suitable.

Apparently dormant lateral buds were excised from shoots of *Populus flevo, P. yunnanensis, P. nigra* Italica, *P. tremoides* and *Alnus viridis*. For alder, juvenile apical and lateral buds were also tested. These buds were excised from shoots of one year old seedlings maintained in growth cabinets.

After 2 weeks of incubation on medium 1 (M1; 0.2 mg/ℓ BA), bud break occurred and extension growth of the preformed embryonic shoot had commenced in all species, but at apparently different rates. Alder consistently showed little bud burst and shoot extension, whereas bud explants of *Populus* species grew readily. Axillary buds on the extending embryonic shoot of *Populus yunnanensis* had also started to grow. With all species, but more so for *Alnus*, the establishment of bud explants free from microbial contamination was a problem. From the initial explants, 20 per cent of the *Populus* sp. and 65 per cent of the *Alnus* species showed signs of contamination. Although contamination was less (45%) for juvenile tissue of alder, the number of buds that survived the sterilization procedure (section 2.6) was less, and thus the number of bud explants that remained was approximately the same for juvenile and dormant tissue. The contaminated bud explants and those that did not show visible signs of shoot extension were discarded. For each of the remaining bud explants, the surrounding residual dead tissue was separated from the young tissue and the buds transferred back on to M1.

Following 4 weeks of culture on M1, a visual score was made of the number of adventitious buds that had developed for each bud explant (Table 3.36). The *Populus* species consistently showed signs of adventitious bud formation and proliferation on cut and uncut surfaces. There was evidence of callus formation at or near the tissue submerged in the medium. The occurrence of adventitious buds was greater on shoots of *Populus yunnanensis* and *P. tremoides* than *P. flevo*. No adventitious buds could be seen on the alder shoots, although some extension of the embryonic shoot had taken place and callus formation was evident at the shoot base. Generally, alder showed signs of senescence at the base of the shoot after 3 weeks, and separation of the apical portion from the senescing basal portion was necessary for continued growth at the apex. In addition to M1, a range of other media were also unsuccessful in inducing multiple bud development in alder bud explants (Table 3.36).

Table 3.36 The effect of medium composition on the formation of adventitious buds from bud explants of *Alnus viridis* and four *Populus* species. Values represent the mean number (± S.E.) of adventitious buds per explant. Ten explants were scored per treatment and each treatment replicated three times.

	Media				
	½ MS	Full MS			
Growth substances mg ℓ^{-1}	M1 (0.2 BA)	M0 (0.5 BA)	M1 (0.2 BA)	M2 (0.1 BA/ 0.02 NAA)	M3 (0.2 BA/ 0.02 NAA)
Alnus viridis					
dormant buds	0	0	0	0	0
juvenile buds	0	0	0	0	
Populus yunnanensis	n.t.	n.t.	>20 ± 4	n.t.	n.t.
P. nigra	n.t.	n.t.	> 8 ± 3	n.t.	n.t.
P. tremoides	n.t.	>20 ± 6	>20 ± 4	n.t.	n.t.
P. flevo	n.t.	n.t.	>12 ± 3	n.t.	n.t.

n.t. = not tested
MS = "Murashige and Skoog medium" given in section 2.5.

Apparently single buds were excised or separated from the parent mass that had developed, and transferred on to medium containing 0.02 mg ℓ^{-1} NAA and 0.1 mg ℓ^{-1} BA (M2). After 4 weeks of incubation, the number of buds that produced shoots of 40 to 60 mm in length were counted (Table 3.37).

Table 3.37 The effect of medium composition on shoot development from apparently single adventitious buds of four *Populus* species or from bud explants of *Alnus viridis*. Values represent the percentage of buds that developed into shoots greater than 40 mm in length. Each treatment consisted of 100 adventitious buds (*Populus*) or 100 bud explants (*Alnus*).

	Media		
	M1	M2	M2 + GA_3 (1 mg ℓ^{-1})
Alnus viridis			
dormant buds	0	0	0
juvenile buds	0	0	0
Populus yunnanensis	3	60	n.t. (not tested)
P. nigra	0	22	n.t.
P. tremoides	1	39	n.t.
P. flevo	n.t.	36	n.t.

Alder failed to produce any shoots greater than 8 mm in length whereas all *Populus* species produced shoots greater than 40 mm in length. From each single bud cultured, a number of shoots usually developed but in most cases only one or two shoots were greater than 40 mm in length. Similarly, where several adventitious buds that had not been separated and were cultured together, several shoots greater than 40 mm in length were readily obtained with *Populus* species.

It was decided to use *Populus yunnanensis* in subsequent studies because of the rapid production of a substantial number of uniform shoots.

3.5.2 Description of Culture System and Growth

Despite the excision and culture of apparently single buds, the development of a single bud into a single shoot was not realised on all occasions when buds were incubated on M2 (Plate 1). The tendency for callus formation and adventitious buds to form and proliferate on cut and uncut surfaces resulted in multiple shoot formation. The resultant growth consisted of the development of several shoots, generally 2 or 3, and some adventitious bud formation and proliferation from the callus formed at bases of the excised buds or newly formed shoots (labelled X on Plate 1). Single shoots with some callus formation only but no adventitious bud formation (labelled Y on Plate 1) were observed to occur less frequently. Some of the single buds failed to show any shoot development but adventitious bud development and proliferation occurred readily in these buds (labelled W, Plate 1). The response of these buds was similar to buds incubated on M1. The resultant growth consisted entirely of the development of adventitious buds with their respective leaves but no shoot extension.

It was not always possible to have the ideal of a single growing shoot, consisting of the apex plus 2 or 3 leaves, without callus formation and adventitious bud development. However, to achieve this ideal, all buds were excised from tissue that had been growing on M2 for three weeks previously. The excised shoot tip (apex plus 2 or 3 leaves) was then transferred back on to M2. At this stage, most shoot tips were 5 mm to 8 mm long and had fresh weights of 10 to 15 mg. After 1 week of incubation, those shoots showing the least callus formation and/or adventitious bud development, but the greatest growth,

were used for the bioassay of growth regulators. However, multiple shoots were used in the photoperiod response studies.

It was necessary to culture approximately 250 single shoots to get 100 apparently uniform shoots.

3.5.3 Response to Photoperiod

The growth response of aseptically cultured shoots to a change in photoperiod from LD's to SD's was recorded at regular intervals (Table 3.38).

Table 3.38 The effect of photoperiod on the growth of aseptically cultured shoots of *Populus yunnanensis*. LD's represent 16 hour photoperiods and SD's 8 hour photoperiods. Values represent means ± standard errors of three replicates, each consisting of shoot growth in ten individual culture tubes.

Photoperiod	Day 0	Day 7	Day 21
LD's			
Fresh weight (mg)	737.9 ± 122.1	793.4 ± 193.6	822.1 ± 124.8
Dry weight (mg)	34.8 ± 2.2	32.2 ± 1.35	37.9 ± 3.1
Length (mm)	21.0 ± 2.5	24.9 ± 2.25	27.3 ± 2.0
SD's			
Fresh weight (mg)	792.8 ± 70.5	763.6 ± 111.8	695.3 ± 124.8
Dry weight (mg)	37.0 ± 2.0	34.2 ± 1.8	31.4 ± 3.5
Length (mm)	22.9 ± 0.9	23.9 ± 2.3	23.3 ± 3.6

Sets of 2 to 3 buds were excised from shoots maintained on M2 and then transferred back on to M2. The multiple buds were incubated on M2 for 3 weeks under LD's and then transferred to SD's. The fresh weights and dry weights represent the growth of multiple shoots from the excised buds, whereas lengths represent the longest shoot only in each set of multiple shoots. After 3 weeks, there were no significant differences ($p > 0.05$) between the growth of shoots maintained under LD's and shoots maintained under SD's, although the fresh and dry weights and length were higher for the LD treated shoots. However, marked differences in the morphology of the apical region were apparent (Plate 2). In SD treated shoots, internode extension in the region of the apex had been suppressed, and hence the leaves appeared to be originating from the from the same node at or near the apex (Plate 2b). No new emerging

leaves could be seen in the apical region of SD shoots, whereas new leaves were being produced and clearly seen on LD shoots (Plate 2a). Internode extension at the apical region was also visible on the LD shoots, and individual nodes could be easily distinguished. Within each set of multiple shoots maintained under SD's, only the longer shoots consistently showed the change in morphology at the apical region. Smaller shoots, less than 10 mm long, did not appear to have ceased growth. It was assumed that only some apical buds on the SD shoots were dormant. Excision and transfer of 10 apparently dormant apices back on to M2 and under a LD photoperiod (16 hour) did not result in any visible shoot growth, although callus formation was obvious at the shoot bases.

3.5.4 Assay for Dormancy-Inducing Substances

The existence of dormancy-inducing substances in the acidic ether-soluble fraction of plant extracts was tested. Leaves and apices from dormant plants were harvested separately and the extracts were replicates of those used for inhibitor β studies (details in sections 2.9.1 and 2.9.2). Each extract was fractionated by paper chromatography as for the inhibitor β studies. However, in this case the chromatograph was divided into ten equal R_F sections, which were assayed on aseptically cultured shoot tips. The growth response, i.e. change in fresh weight and length, was measured and any indication of dormancy noted. In addition to the acid ether-soluble fractions, the 80% MeOH extractable fraction (termed crude extract) of aseptically cultured shoots, grown for 4 weeks under SD's, was also tested.

3.5.4.1 Leaf extract

The leaf extracts were assayed at serial dilutions equivalent to 1.0, 0.5 and 0.25 g dry weight of leaf material. Two extracts were assayed. The first, termed "outside" extract, was of leaves from trees that had entered dormancy under natural conditions and represented a replicate of H3 extract, 1979 (see section 2.9.2). The second extract, termed "inside" extract, was of leaves from seedlings maintained under SD's and was a replicate of H2 extract (see section 2.9.1) in the photo-induction experiment.

The growth response of the shoots varied between R_F sections of chromatographs of the "outside" extract (figures 125 and 126 for fresh

weight and length, respectively) and the "inside" extract (figures 127 and 128). Generally, the growth response between replicate (3) chromatographs was extremely variable as indicated by the large standard errors, and therefore, any specific comparisons between R_F sections within a chromatograph are most likely to be non-significant. The variable nature of the response (length and fresh weight) occurred at all dilutions of both extracts.

The assay of the control chromatographs also resulted in very variable growth responses between replicates, and hence, statistical comparisons between control and test chromatographs were not possible on an individual R_F basis. In most cases, the shoot response to the presence of the control R_F section and the test R_F section were not markedly different and their respective standard errors overlapped. Despite the variable nature of the growth response, some trends were observed.

Most R_F sections were inhibitory to shoot growth when compared to growth in their absence, i.e. growth of the blank control (figures 125 and 126). The visual growth response of shoot tips to the inhibitor β fraction of either extract was consistently less than the growth response of the blank control, and this was to some extent reflected by the fresh weight and length determinations (figures 125-128). On the other hand, the R_F zone 0.0 to 0.2 on chromatographs of "inside" extract, assayed at the highest dilution, consistently showed a promotory effect on shoot growth (figures 127 and 128). Similarly, a zone of growth promotory activity was evident at R_F 0.3 to 0.5 on chromatographs of "outside" extract assayed at the equivalent of 0.5 g dry weight leaf material (figures 125 and 126).

None of the R_F sections assayed resulted in the appearance of shoot morphology characteristic of dormant buds and shoots. The formation of new leaves was observed on all shoots.

3.5.4.2 Apical extract

An extract of dormant apices was assayed at dilutions equivalent to 1.0, 0.5 and 0.25 g dry weight of apical material. The extract was a replicate of H3, 1979 (see section 2.9.2) and represented apices which had entered dormancy under natural conditions.

The assay of chromatographs of extract resulted in markedly different growth responses at different R_F's (figures 129 and 130, fresh weight and length, respectively). At all dilutions, there was considerable variability between the replicates and for most R_F sections, the standard error of the mean associated with replicate fresh weight and length determinates were large and overlapped with the estimates of other R_F sections. The variability between standard errors made any statistical comparisons invalid.

The large variability between replicates also occurred between replicates of control chromatographs (figures 125-128 and 129-130). This unexpected result, when considered alongside the data of the control chromatographs in the leaf extract study, suggested the variability was associated with the tissue and not the chromatographs. When test chromatographs are compared with control chromatographs, the growth responses of the respective R_F sections overlap when standard errors are also considered, and therefore, no differences are likely to be significant. Nevertheless, the R_F zone 0.5 to 0.8 consistently resulted in less apparent (visual) growth, although the specific fresh weight and length values are not markedly different at all dilutions.

No dormancy-inducing substance(s) was detected on any chromatograph of apical material at any of the dilutions assayed.

3.5.4.3 Crude extract

After reducing the 80% MeOH extract to aqueous, the crude extract was assayed on growing shoots at serial dilutions equivalent to 0.025, 0.25, 2.5 and 25 mg dry weight of shoot material.

At the highest dilution assayed, the growth of the shoots was markedly affected by the presence of crude extract in the media (figure 131). Fresh weights and lengths of shoots were markedly less in the presence of crude extract than its absence. At the three lower dilutions, fresh weights of shoots were consistently higher than but not markedly different from the fresh weights of control shoots. Shoot length was comparable to that of the control shoots.

Although growth was consistently less in the presence of crude extract, the apparent cessation of growth did not occur. The formation of new leaves was apparent at the apex of all shoots.

The results suggested that no dormancy-inducing substance(s) was detected by the aseptically cultured shoots.

3.5.5 Effect of Growth Regulators

The growth response of aseptically cultured shoots to the presence of growth regulators; ABA, AMO 1618, GA_3, ABA + AMO, C5 and C10, at various concentrations in medium 2, was tested. After 6 weeks of incubation, the growth responses of shoots differed markedly for each of the growth regulators. The results are presented as dose-response curves.

3.5.5.1 Abscisic acid

The addition of ABA to M1 and M2 resulted in less growth in shoots incubated on these media. Abscisic acid was added to the media in quantities that gave final concentrations equivalent to 0.01, 0.1, 1.0 and 10 mg ℓ^{-1}.

Shoots growing on M1 in the absence of ABA showed considerable adventitious bud formation and proliferation (Plate 3). The presence of increasing quantities of ABA in the medium, resulted in less apparent adventitious bud development and this was reflected in the fresh weights determined. Shoot fresh weight was consistently less in the presence of ABA at all concentrations than in its absence (figure 132). At the lowest concentration (0.01 mg ℓ^{-1}) a small non-significant depression of shoot fresh weight occurred, whereas in the presence of all other concentrations a significant difference ($p < 0.05$) in fresh weight was found. Inhibition of shoot extension also occurred, although the shoots incubated on this medium, naturally show little shoot extension. The decrease in fresh weight with increase in ABA concentration was approximately a logarithmic function.

Shoot growth was markedly affected by the presence of ABA in M2 (Plate 3, figure 132). The fresh weights and lengths of shoots were less in the presence of ABA at all concentrations. However, shoot length was only significantly less ($p < 0.05$) in the presence of ABA at the highest concentration (10 mg ℓ^{-1}), whereas the effect on shoot fresh weight was significant at concentrations greater than 0.1 mg ℓ^{-1}. The decrease in shoot growth with increase in ABA concentration resulted in a dose-response curve which was approximately logarithmic.

In the presence of the highest ABA concentration, the leaves on some shoots turned yellow and for all shoots, little or no shoot extension took place. However, a newly formed leaf could be seen emerging from the apical region of these shoots and following transfer of these shoots on to an ABA free medium, further extension and development of the newly formed leaf and internode occurred.

The results suggested ABA inhibited the formation and proliferation of adventitious buds and stem extension but did not cause growth cessation in shoots or induce bud dormancy.

The stability of ABA during autoclaving was checked qualitatively and semi-quantitatively by U.V. spectroscopy. If optical density (OD) is taken as a measure of the stability of ABA, then the OD at λ_{max} would be expected to be less, and possibly together with the appearance of other peaks at different wavelengths if any degradation of ABA had occurred. No apparent differences in the U.V. spectra of autoclaved and non-autoclaved ABA were noted (figure 133). The OD's at $_{max}$, which was 248 nm for aqueous ABA and 263 nm for ABA in EtoAc, were considered to be within experimental error. The λ_{max} agreed with that given in the literature by Wilmar and Doornbos (1971), Hall (1978) Milborrow (1967) and Jenkins and Shepherd (1972).

It was concluded ABA did not undergo excessive conversion or degradation during autoclaving.

3.5.5.2 AMO 1618

The presence of the plant growth retardant AMO 1618 had an inhibitory effect on the growth of aseptically cultured shoots incubated on M2 (Plate 4). The dose response was approximately logarithmic when AMO 1618 was added to M2 at concentrations equivalent to 0.01, 0.1, 1.0 and 10 mg ℓ^{-1}. At all concentrations, shoot fresh weight was inhibited, whereas shoot length was inhibited only when AMO 1618 was present at a concentration greater than 1 mg ℓ^{-1} (figure 134). The inhibition of shoot growth in the presence of 10 mg ℓ^{-1} AMO 1618 was comparable to that of ABA at 1 mg ℓ^{-1}. Although the extension of stems was inhibited, the formation of new leaves at the apex was still observed, indicating that bud dormancy had not been induced.

3.5.5.3 ABA + AMO 1618

The combined presence of ABA and AMO 1618 at dilutions that gave final concentrations of 0.01, 0.1, 1.0 and 10 mg ℓ^{-1} resulted in a marked reduction on the growth of shoots (Plate 5). The response to the combined solution was comparable to the presence of ABA alone, but greater than in the presence of AMO 1618 alone. Increase in shoot fresh weight was consistently inhibited at all concentrations whereas shoot length was not markedly affected (figure 135). At the highest concentration, the inhibitory effect on shoot fresh weight and length was significant ($p < 0.05$) and most shoots achieved little or no growth during the incubation period. However, the apical buds on these shoots were considered not to be dormant as new leaf production was still apparent. The transfer of five of these buds on to M2 resulted in continued shoot growth.

3.5.5.4 Gibberellic acid

The growth response of shoots in the presence of GA_3 was dependent on the concentration present in the medium (Plate 6). In the presence of GA_3 at 10 mg ℓ^{-1}, the growth of shoots was promoted, whereas at lower concentrations a significant decrease in shoot growth occurred (figure 136). At the highest GA_3 concentration, 10 mg ℓ^{-1} shoot fresh weight was increased by approx. 50%, whereas an approx. two-fold increase in shoot length had occurred. The increase in shoot length appeared to be the result of greater internode extension rather than the production of new nodes. Those concentrations, 0.01, 0.1 and 1 mg ℓ^{-1}, that inhibited shoot fresh weight and length did not induce the formation of dormant buds.

3.5.5.5 C5

From the analysis of two independent experiments it was found that the presence of C5 did not produce a consistent affect on the growth of aseptically cultured shoots. The data from one experiment suggested that C5 at concentrations equivalent to 1.0, 10, 50 and 100 mg ℓ^{-1} had little effect on shoot length and fresh weight, although at 100 mg ℓ^{-1} these growth parameters were consistently less than in the absence of C5 (Plate 7, figure 137). However, when the experiment was repeated, the data suggested that shoot length was increased in the presence of C5 whereas shoot fresh weight remained the same, except in

the presence of C5 at 10 mg ℓ^{-1} when shoot fresh weight was increased. None of the differences between the growth parameters in the presence and absence of C5 was considered to be significant.

In both experiments where shoot fresh weight and length were lower in the presence of C5, all the shoots appeared to be growing and no obvious signs of growth cessation or dormancy were associated with these shoots.

3.5.5.6 C10

Decanoic acid was added to M2 at concentrations of 1.0, 10, 50 and 100 mg ℓ^{-1}. The growth response of shoots in the presence of C10 was dependent on the concentration present. At the lowest concentration tested, C10 inhibited shoot fresh weight and length whereas at the highest concentration, an increase in these growth parameters occurred (figure 138). The maximum inhibitory effect of C10 occurred at 10 mg ℓ^{-1} but this effect was not comparable to ABA. The promotory effect on shoot fresh weight appeared to be the result of the stimulation of adventitious bud production and proliferation, and only a slight stimulation of stem extension (Plate 8).

There was some discrepancy between the results of the replicate experiments, especially at the highest concentration tested. In one replicate experiment, shoot fresh weight was not affected although some increase in shoot length did occur (figure 138).

The inhibitory growth response of C10 did not result in the formation of dormant buds. Most shoots were observed to be forming new leaves although stem extension was markedly less.

3.5.6 Sugar and Starch Content of Aseptically Cultured Shoots

The sugar-starch content of aseptically cultured (on M2) shoots growing in the presence and absence of ABA at concentrations of 0.01, 0.1, 1.0 and 10 mg ℓ^{-1} and GA_3 at 10 mg ℓ^{-1} was determined. Total soluble sugars and starch were determined on the same sample and the data expressed as a percentage of the dry weight of tissue extracted.

Care was taken to ensure that all shoots and tissues were thoroughly cleansed and rinsed of any residual agar to avoid sucrose contamination from the medium.

In the presence of ABA at all concentrations, total sugar content of shoots was consistently higher than in its absence (Table 3.39). In the absence of ABA, the total sugar content was 8.21% of the dry weight, whereas in the presence of 10 mg ℓ^{-1} ABA the mean total sugar content was 13.6%. However, the differences between the total sugar content of shoots in the presence and absence of ABA at all concentrations was not significant ($F = 2.8974$, df 4, 13; $p > 0.05$).

Table 3.39 The effect of ABA and GA_3 on the total soluble sugars and starch content of aseptically cultured shoots of *Populus yunnanensis*. Values represent percentage sugar or starch (± standard error) per unit dry weight of tissue. Each treatment comprised 10 shoots and was replicated three times.

Treatment	Sugar		Starch		Ratio
M2 (control)	8.2 ± 1.7	11.10 ± 1.78	0.15 ± 0.048	0.148 ± 0.044	52:1
ABA: 10 mg ℓ^{-1}	13.6 ± 2.96		0.201 ± 0.059		68:1
1.0 "	10.7 ± 0.61		0.127 ± 0.034		84:1
0.1 "	9.4 ± 1.22		0.097 ± 0.074		97:1
0.01 "	10.7 ± 4.06		0.150 ± 0.060		71:1
GA_3: 10 mg ℓ^{-}	6.1 ± 0.8		0.257 ± 0.034		24:1

In the presence of GA_3 at 10 mg ℓ^{-1}, the total sugar content was markedly decreased to 6.1% and this was significantly different ($p < 0.01$) from the total sugar content (8.2%) of untreated shoots.

The starch content of shoots was not markedly affected by the presence of ABA at all concentrations tested (Table 3.39). In the presence of ABA, over all concentrations, the mean starch content was 0.148% whereas in the absence of ABA the starch content was 0.157%. At the highest concentration tested, ABA increased the starch content to 0.201%, whereas in the presence of ABA at the lowest concentration, starch content was much lower (0.150%) and comparable to the control. However, there were no significant differences between the various ABA treatments.

The starch content of shoots incubated in the presence of GA_3 was higher than shoots incubated in its absence. The difference between

the starch contents was significant ($p < 0.05$).

The sugar:starch ratio was not markedly affected by the presence of ABA, but GA_3 caused a reduction in the ratio from 52:1 to 24:1.

The results suggested ABA did not have a significant effect on the starch and total sugars content of shoots, whereas the total sugars content was significantly decreased and the starch content increased by GA_3. The total sugars content was, however, consistently higher in the ABA treated shoots.

CHAPTER 4

DISCUSSION

Investigations into the hormonal regulation of bud dormancy of woody species led to the discovery of abscisic acid and the establishment of a role for ABA in the induction and maintenance of bud dormancy (Wareing, 1969; Wareing and Ryback, 1970; Wareing and Saunders, 1971; Wright, 1975). In the present study, no unambiguous evidence in support of a role for ABA in the regulation of bud dormancy was found. Endogenous levels of free and bound ABA in apices and leaves were not correlated with the induction of dormancy, and exogenously supplied ABA was unable to induce dormancy in buds of actively growing shoots nor prolong the dormancy of dormant buds.

Many woody plants form terminal buds under short photoperiods, and it has been widely believed that ABA in shoot tips increases during short photoperiods, leading eventually to the formation of terminal buds (Eagles and Wareing, 1964). The results of experiments in this study fail to support this concept. The transfer of photoperiodically sensitive *Alnus viridis* seedlings from LD's to SD's did not cause a rise in the amount of ABA present in methanolic extracts of apices and expanded leaves. Several other investigations in recent years have also shown that the ABA content does not increase in shoot tips of woody plants under short day conditions (Lenton et al., 1972; Loveys, Leopold and Kriedemann, 1974; Powell, 1976; Alvim et al., 1979). The absence of a rise in ABA content during photoinduction of bud dormancy argues against a role for ABA in the natural induction of dormancy. However, it is possible that there is an interaction between ABA content, photoperiod and temperature, and the absence of a rise in ABA content is attributable to the high night temperatures or constant day night temperatures used in the above studies. In the present study, a low night temperature ($10^{o}C$) comparable to that expected under natural autumn conditions was used, and these results suggest that high night temperatures used in previous studies can not explain the absence of a rise in ABA content.

Since in the present studies, the specific technique of quantitative gas chromatography, in which corrections for losses of ABA

were made and used to determine the ABA content of extracts, it is unlikely that a SD-induced rise in ABA content did occur but remained undetected. The possibility of not detecting a rise in ABA content exists in cases where analysis is by bioassay methods because of the possible problems of a lack of sensitivity and specificity. Such problems are not expected when quantitative gas chromatography is used. However, it was shown in the present study that the specific technique of gas chromatography is not beyond question. Confirmation of the identity of the putative ABA peak by co-chromatography and the technique of U.V. isomerisation may be inadequate and lead to erroneous results and subsequent interpretation. In the case of trans, trans Me-ABA it was found that trace quantities did exist in purified extracts, but quantitication was not possible because of the presence of a compound with a similar retention time. Phillips et al. (1979, 1980) also report the existence of such a compound and proposes it is phaseic acid, a metabolite of abscisic acid, but this was not confirmed in the present study. Nevertheless, the presence of such a compound raises doubts on the use of trans, trans ABA as an internal standard for the correction of losses of ABA during extraction and purification as recommended by Lenton et al. (1971). Furthermore, the presence of the interfering compound(s) casts doubt on the existence and levels of trans, trans ABA present in bud tissues reported by Leshem et al. (1974) and Jones et al. (1976). These two groups of investigators have suggested that the decreasing presence of the trans trans stereoisomer is the major factor in the mechanism of bud break, although the cis trans isomer was also present in buds. In view of the present results, in which it was shown retention times and co-chromatography are insufficient for identification of the trans, trans isomer, such a concept of bud break may be erroneous. Jones et al. (1976) used mass spectrometry, in addition to co-chromatography and comparison of retention times, to confirm the identity of trans, trans ABA although no data was presented. Unless a quantification method, e.g. single ion monitoring (S.I.M.) (Horgan, 1980), was also attempted, it does not necessarily follow that the gas chromatography peaks found are caused entirely by the compound for which a mass spectrum was obtained. The mass spectrum of a compound provides evidence of its presence and identity only.

The technique of U.V. isomerization is widely used for confirmation of the presence of ABA in extracts. However, in this study

and those reported by Plancher (1979) and Dumbroff, Cohen and Webb (1979), it was found that a 1:1 ratio of cis,trans to trans,trans ABA is not always possible. Isomerization of ABA was accompanied by a loss in total ABA due to degradation and this resulted in the formation of several extraneous peaks at the same time as the disappearance of the cis,trans ABA peak and the appearance of the trans,trans ABA peak, when the U.V. treated solutions were gas chromatographed. Therefore it is possible that extraneous peaks may or may not represent compounds originating from the present Me-ABA. In view of this, the use of U.V. isomerization for the confirmaation of identity is limited to those cases where a 1:1 ratio is obtained and no significant loss in ABA occurs.

Despite some doubts concerning the use of direct chemical methods for ABA analysis, it does appear that SD treatment has no effect on the ABA content of leaves and developing or growing buds. In this study a gas chromatograph equipped with an electron capture detector was used for ABA determinations, and corrections for losses during purification were determined. The levels of free ABA found in mature, expanded leaves and apical buds (approx. 188 ng kg^{-1} and 480 ng kg^{-1} dry weight of tissue, respectively) are comparable to levels recorded for tissues of woody species (Milborrow, 1978a). To make comparisons between ABA levels and compensate for the morphological changes that occurred at shoot apices during the photoinduction of bud dormancy, the ABA levels determined were expressed as ng kg^{-1} dry weight of tissue. This, of course, reflects the relative amount of ABA present per unit of tissue and does not necessarily reflect the effective concentration present in tissue. Although not attempted in the present study, Lenton et al. (1972), Alvim et al. (1978) and Phillips et al. (1979) have all shown that on a per bud basis and on a fresh weight basis, there is no increase in the ABA content of buds (and leaves) following their transfer from LD's to SD's. Originally, Wareing (1969) proposed that ABA was synthesised in the leaves under the influence of SD's and then transported to the growing apices where it caused resting bud formation. The concentration of ABA found in the leaves and apices in the present study and that of Lenton et al. (1972), indicates that growing apices already have a relatively higher ABA content than leaves. If ABA is acting solely as a growth inhibitor, the presence of such high levels in the apices is difficult to explain. The plants used in this study were free of any observable stress, e.g. water, that is known to increase

the endogenous ABA content of tissues (Zeevaart, 1979; Wright, 1978; Walton, 1980). However, the unexpectedly high amounts in growing buds may be of little significance in view of the possible compartmentation of ABA within the cell (Loveys, 1977; Milborrow, 1979; Heilmann, Hartung and Grimmler, 1980; Hartung, Gimmler, Heilmann and Kaiser, 1980). Therefore, it is possible that the SD induction of dormancy involved a change in the rate of transport of ABA from the leaves to the apices, together with a change in the rate of synthesis, degradation and cytoplasmic release within the two tissues rather than a change in absolute amount or concentration of ABA present.

Irrespective of the nature and accuracy of the quantitative analysis, the possibility remains that a SD induced rise in ABA content did occur but was not detected. The ability to detect such a rise may be a function of the frequency of sampling and the nature or part of the plant sampled. If only a transient rise in ABA content is required for the induction of dormancy then sampling of tissue, as carried out in this study (approx. weekly intervals), may not be sufficient to observe the rise. With a high frequency of sampling, a transient rise in ABA content is more likely to be detected, whereas weekly sampling of tissue only enables a rise in ABA content of much greater duration in time to be observed. To compensate for the artificial situation of a sharply reduced photoperiod (growth cabinet studies), in contrast to the natural situation of a progressive reduction in photoperiod, the plant may well resort to a transient rise in ABA content or alter the release and metabolism of ABA to achieve the induction of dormancy through the action of ABA. Furthermore, the transient rise in ABA content may occur in some parts of the plant and cells only. However, it is unlikely that a transient rise occurs, as under natural conditions it has been found that the ABA level in buds increases gradually during the autumn, reaches a peak and then gradually disappears during spring (Saunders et al., 1974; Wright, 1975; Alvim et al. 1978; Mielke and Dennis, 1978; Dumbroff, Cohen and Webb, 1979). Furthermore, the level of ABA present in the buds shows a strong correlation with the growth and dormancy of the bud; the level of ABA is low in growing apical tissue and high in dormant tissue. Although, under the experimental conditions in this study, the transfer from LD's to SD's was abrupt, it was expected the ABA content of leaves and buds would also show an increase and remain at this higher level as the buds became dormant. In a study by Alvim

et al. (1979), where daylength was progressively shortened, it was shown that the ABA content of shoot tips remained unchanged. Therefore, it does not seem possible that the failure to sample at an appropriate photoperiod and frequency, or the abruptness of the transfer from LD's to SD's, is the reason for not observing a rise in the ABA content of apices and leaves as predicted by the inhibitor concept of ABA and its regulation of bud dormancy.

In this study, no evidence was found for photoperiodically induced dormancy to be associated with a change in the metabolism of ABA. The level of the biologically active form of abscisic acid (the cis, trans isomer) as well as the inactive bound form (presumed to be the glycosyl ester of ABA) remained unchanged. However, in this study bound ABA was obtained by alkali hydrolysis of extracts, and therefore, excluded any conjugates not capable of being hydrolysed as well as any ABA that was bound to plant tissue or cell constituents. The relative importance of other conjugates and cell bound ABA as storage products or as inactivation mechanisms is unknown. It has been assumed that the glucose ester of ABA is a biologically inactive derivative from which ABA as the free acid can be released if and when necessary (Hiron and Wright, 1973; Milborrow, 1974; Harrison and Saunders, 1975; Wright, 1975; King, 1976). However, most recently Milborrow (1978b) has raised some doubts as to whether ABA in the form of the glucose ester is actually a storage (and therefore, inactive) form. In tomato and silver beet shoots, Milborrow (1978b) found that the conjugation of ABA was irreversible, and therefore, the pool of sequestered ABA as alkali-hydrolysable conjugates, including the glucose ester, may not be reused. His data suggested ester formation sequesters ABA permanently, and therefore, the glucose ester is not a storage form but a degradation product. On the other hand, in barley shoots Lehmann and Schutte (1981) who worked specifically with the glucose ester of ABA, in contrast to Milborrow (1978b) who studied the alkali hydrolysable conjugates, found the *in vivo* hydrolysis of the ABA glucose ester does occur. In the present study, the ABA released from apical extracts upon alkali hydrolysis was approximately the same as the amount of free ABA, whereas in leaf extracts the amount was approximately one-third the quantity of free ABA. The amount of ABA released by alkali treatment is usually between one-tenth and one-third of the amount existing as free acid (Lesham, Philosoph and Wurzburger, 1974; Milborrow, 1974;

Powell and Seeley, 1974; King, 1976), but in some tissues (Goldschmidt, Goren, Even-Chen and Bittner, 1973; Wright, 1975) the conjugated ABA equals or exceeds the amount of free acid. The levels of alkali hydrolysable ABA determined in this study are open to question in view of the findings of Zeevaart (1980). He reports that the use of ethyl acetate during the solvent partitioning step in the purification procedure can result in an underestimate of the amount of ester present. Nevertheless, the present study does show that the contribution of the inactive form to the total pool of ABA present in tissues can vary considerably, and presumably this enables the different tissues to regulate their own levels of free or active ABA. However, SD-induced dormancy does not appear to be associated with a change in the relative amounts of free and ester forms or alkali hydrolysable forms present.

Given that the ABA content of tissues does not change during photoinduction, a role for ABA in the induction phase of dormancy is not necessarily ruled out. It is possible that the sensitivity of the apical tissue to ABA is altered under SD conditions, and therefore, a rise in the ABA content or a shift in the metabolism is not necessary for the initiation and progress of dormancy. Furthermore, little is known of the rates of ABA synthesis and inactivation, compartmentation, and the removal and transfer to receptor sites during the initiation of dormancy, and therefore, the evaluation of these aspects of ABA activity may yet reveal a role for ABA in the induction of dormancy. Nevertheless, if ABA levels do not increase under the influence of SD's, the role of ABA as a dormancy must be questioned. On the basis of the present findings and those of Lenton et al. (1972), Loveys et al. (1974), Powell (1976), Alvim et al. (1979) and Phillips et al. (1980), it appears that endogenous ABA in roots, leaves and buds does not have a direct controlling effect on the formation of resting buds. It is difficult to envisage a controlling system for bud dormancy in which the changes in the concentration of free and bound ABA alone regulate the initiation and progress of dormancy. However, these observations do not preclude a role for free and bound ABA in controlling other aspects or phases of dormancy.

When trees growing under natural conditions are considered, the case for a causal role of ABA in the inception of the winter dormant period is strong. The basis for such a role is the evidence from

investigations where changes in ABA content of shoot tips and buds throughout the year were determined. The ABA content of tissues increases in late summer and early autumn during the initiation of dormancy and then gradually decreases in the winter and during the time of bud burst in the spring (Davison and Young, 1974; During and Buchmann, 1975; During and Kismali, 1975; Harrison and Saunders, 1975; Mielke and Dennis, 1975b, 1978; Wright, 1975; Alvim et al., 1976; Emmerson and Powell, 1978; Seeley and Powell, 1981). Although there are exceptions, e.g. Mielke and Dennis (1975b), the ABA peak and the intensity of dormancy are well correlated. However, the correlative evidence just cited raises two questions with respect to the role of ABA in regulating shoot growth:

(1) Is the increase in ABA causally related to the cessation of shoot growth and the increasing intensity of the dormant condition seen in late summer and autumn?

(2) Is the disappearance of ABA during the winter connected with the emergence from the dormant condition?

In answer to the first question, and arguing against an inhibitory role for ABA regulation of growth, is the evidence from this study that the highest concentration of ABA was found in the apices and shoot tips of growing shoots and not the expanded mature leaves. The occurrence of higher levels of ABA in shoot tips than in mature leaves has also been reported by Powell (1975a,b), Sengupta, Rogers and Lorah (1974), and Sweetser and Vatvars (1976). Similarly, the levels of ABA-like substances in shoot tips and axillary buds of intact *Phaseolus vulgaris* plants were the same, and there was only a slight drop in the levels in axillary buds 24 hours after decapitation of the main shoot, by which time the outgrowth of the bud had commenced (White and Mansfield, 1977). If ABA is a powerful inhibitor of growth, then it would be expected that in elongating shoots the concentration would be very low and not the reverse as was found. Therefore, it is questionable whether ABA is acting as a growth inhibitor in growing shoots. As discussed above, compartmentation of ABA is possible and it would appear that it would be a necessary prerequisite if the inhibitor concept of ABA is to be held for growing shoots of woody species. It may be of significance that ABA has been shown to act as a promoter of growth (McWha and Jackson, 1976).

With regard to the question concerning the decrease in ABA and the emergence from dormancy, it is noted that most but not all experiments report a gradual decrease in ABA content as winter and chilling proceeds. In the case of the exceptions, it is possible that a lack of a correlation between the degree of chilling and ABA content was a result of the use of uncontrolled natural temperatures. Temperatures around 5^{o} to $7^{o}C$ are most suitable for chilling, and under higher or lower temperatures chilling efficiency is decreased (Richardson, Seeley and Walker, 1974; Campbell and Sugano, 1975; Erez et al., 1979). Therefore, for buds from trees under natural conditions, the ABA content and degree of chilling may not be well correlated because of the wide and erratic fluctuations in temperatures. Some of the conflictory evidence may be explained from the view that there are different states or phases in the development of dormancy, and as winter progresses the dormancy of buds changes. In autumn, buds may be in a state of correlative dormancy whereas in late winter the buds may be in a state of imposed dormancy or quiescence. It is only during late autumn-winter that buds are in a state of innate dormancy, and this phase of dormancy may last for only a short time. Furthermore, the precise timing of these events or phases may vary between species, and therefore, the determination of the exact state of dormancy is of major importance in studies where endogenous levels of ABA have been determined. The lack (or presence) of a correlation between ABA levels and the dormancy state may be explained on the basis that the dormancy state of the buds sampled differed from that expected or assumed. In studies, e.g. Harrison and Saunders (1975), where the dormancy state of the buds was determined, a good correlation exists between the intensity of dormancy (assessed by the time to bud break on transfer to conditions favouring growth) and the level of ABA present in the bud. On the other hand, in many experiments in which the ABA changes are followed during chilling, no attempt was made to determine whether the decline in ABA content, seen during chilling, also takes place in the absence of chilling. Parallel determinations of ABA are seldom made on similar plant material which is held at warm temperatures. The work of Seeley and Powell (1981) and Alvim et al. (1976), in which it was found that the decline in ABA content of buds began well before the onset of chilling temperatures, suggests chilling is not required for the disappearance of ABA. Therefore it is uncertain what role, if any,

winter chilling has in the disappearance of ABA, although the role of chilling in the emergence from dormancy is well established for many woody species. It is possible that chilling has a role in the production of growth promotory hormones such as gibberellins and cytokinins. A correlation between the emergence from dormancy and ABA content of buds can only be suggestive of but not proof of a role in dormancy.

From the above discussion it appears that studies on the endogenous ABA content of shoots and buds from plants growing under natural and controlled environment conditions has not provided conclusive proof of a regulatory role for ABA in bud dormancy. In general, there appears to be an increase in the content of ABA in shoots during summer and autumn followed by a decline in late autumn and winter, but it remains to be proven whether the changes in levels are correlated in a causal sense with growth and dormancy. For the verification of a causal role it is necessary for studies involving exogenously supplied ABA to be made.

A critical experiment to establish a causal role in the induction of dormancy is the application of abscisic acid to actively growing plants which are maintained under conditions promoting active growth. The successful induction of dormancy under these experimental conditions has been reported by El-Antably et al. (1967). In the present study, ABA applied to the leaves and buds did not induce dormancy in alder seedlings and these results are in agreement with those reported by Perry and Hellmers (1973), Saunders et al. (1974), and Hocking and Hillman (1975). Similarly, Cathey (1968) has reported ABA was unable to substitute for SD's in other photoperiodically controlled growth processes, such as stem extension and flowering. It is possible that ABA can exert its effect only when conditions for growth become somewhat less favourable by virtue of lesser amounts of growth promoters, water nutrients and photosynthate. Powell (1975a), Teltscherová and Seidlova (1977), and Hartung and Funfer (1981) have shown that ABA can exert its inhibitory influence more effectively at certain physiological stages than others. It has been suggested (Powell, 1978) that when ABA is applied to plants not in the "grand phase" of growth, growth is inhibited since vigorously growing shoots are little affected by ABA applications whereas weekly growing shoots are more susceptible. This

view is not supported by the observations of the present study. In the case of the isolated shoots (section 3.3.2), where growth was not as vigorous as that observed for shoots left intact on seedlings, the application of ABA still did not result in the formation of dormant buds.

The failure of ABA to induce dormancy in both isolated shoots and seedlings may be attributed to several factors, including slow penetration into the tissue and transport to the active sites, the presence or interference of growth promoters, the metabolism and inactivation, and compartmentation of the exogenously supplied ABA. It has been observed (El-Antably et al., 1967; Hocking and Hillman, 1975) that it is necessary to make repeated applications to the leaves and the apical region directly with ABA to induce inhibition of shoot growth, as only a small proportion of the leaf applied ABA appears to reach the apical region as ABA. Applications of ABA to plants by spraying ABA solutions on to the foliage have been shown to have little or no effect on bud growth, whereas the immersion of a leaf or the cut bases of isolated shoots in an ABA solution has been shown to have some effect (Eagles and Wareing, 1963; El-Antably et al., 1967).

It is unlikely in this study, however, that the failure to inhibit growth or induce dormancy was attributable to the slow penetration and inability of ABA to reach the apex as 10^{-4}M ABA was continually supplied via the leaves (by immersion) and, in addition, applied daily to the leaves and the apical region of seedlings (by spraying and painting). In view of the high concentration of ABA applied and the method and regularity of application, the plants were thought to be saturated with exogenous ABA. Furthermore, when exogenous ABA was supplied through the cut bases of isolated growing shoots of alder, the formation of dormant buds did not occur and no marked inhibition of bud growth was observed. The isolated shoots were observed to take up a substantial volume of ABA solution, and therefore, slow penetration and the inability to reach the apex were not as important a factor as with whole seedlings. Similarly, in studies with aseptically cultured shoots *in vitro*, where growing shoot tips were continuously supplied with ABA, the formation of dormant buds did not occur although shoot extension and the production of new leaves and nodes was markedly inhibited. It appears ABA was able to slow growth considerably but not cause growth to cease. However, in view of no

attempt being made to determine the quantity of ABA actually reaching the apices, the possibility remains that the failure to induce the formation of dormant buds in whole seedlings, isolated shoots and aseptically cultured shoot tips *in vitro* may be due to slow or inadequate penetration of ABA.

It does appear that if enough ABA can be forced into the shoot tip, growth ceases (El-Antably et al., 1967). For example, injections of ABA into apple tree trunks have been successful in causing a reduction or even cessation of growth (Robitaille and Carlson, 1971, 1976; Yadava and Dayton, 1972; Sterrett and Hipkins, 1980). It should be noted, however, that the quantity of ABA injected in these studies was comparable to the amount of ABA taken up by the isolated shoots. Furthermore, excised apple buds cultured aseptically *in vitro* have been shown to readily take up ABA from the basal medium (Singha and Powell, 1978). In the present *in vitro* studies using *Populus* shoot tips, the incorporation of increasing amounts of ABA into the basal culture medium resulted in an increasing inhibition of shoot elongation indicating some uptake of ABA. The concentration of ABA required to induce the formation of dormant buds may be higher than that used in the study. However, the endogenous ABA levels present in tissues of woody plants is in the range 0.01 to 1.0 ppm (Milborrow, 1978a) and the highest ABA concentration tested in this study with shoot tips cultured *in vitro* (10 mg ℓ^{-1}) therefore represents ten times this. That penetration and transport of ABA to the apices did occur is suggested by the observation that shoot elongation increased after inhibited shoot tips were transferred from basal medium containing ABA to ABA free medium. The release from the inhibitory effect of ABA was not as rapid as that observed in citrus bud cultures (Altman and Goren, 1971), apple bud explants (Singha and Powell, 1978) or intact apple plants (Powell, 1975a; Robitaille and Carlson, 1976). Nevertheless, it does appear that continuous supply of ABA is necessary for ABA to have an inhibitory response in woody shoots.

The failure to obtain marked inhibition may also be due to metabolism and inactivation of the applied ABA. It has been shown that woody plants can metabolise exogenously supplied ABA to other compounds (Hocking and Hillman, 1975; Powell and Seeley, 1974; Singha and Powell, 1976). In apple tissue, ABA is assumed to be conjugated with glucose (Powell and Seeley, 1974; Singha and Powell, 1978), and therefore, the

existence of this and possibly other inactivating mechanisms may be responsible for the weak responses to applied ABA observed in the present study. On the other hand, Sterrett and Hipkins (1980) have shown that ABA is not metabolised in dormant apple shoot tizsue, and therefore, it would seem that the age and growth state of the tissue are important factors in the metabolism of ABA. It would, therefore, be necessary to determine the degree of ABA metabolism in each experimental tissue rather than extrapolate from other studies before speculating on the removal or otherwise of ABA from the active pool. Similarly, there is insufficient information to assess the degree, if any, of the compartmentation of the exogenously applied ABA within the cells of apices.

Besides slow penetration, and metabolism and inactivation of ABA, a third possibility exists for the failure to induce dormancy following exogenous applications of ABA. The presence and interaction of growth promoters, such as cytokinins and gibberellins. These growth regulatory substances have been shown to counteract the inhibitory effects of ABA, and vice versa, in several tissues (Wareing, 1978), although for buds and shoots of actively growing woody plants there is little information. In elongating apple shoots, injections of GA_3 overcame the inhibition induced by ABA injections (Robitaille and Carlson, 1976) and in dormant apple shoots, injections of benzyladenine overcame the inhibitory effect of ABA on bud break (Sterrett and Hipkins, 1980). Similarly, Eagles and Wareing (1964) demonstrated an interaction between an inhibitor (presumed to be ABA) and GA_3 in the emergence from dormancy of *Betula* buds. Evidence of the kind just cited, together with reports (cited in sections 1.4.3 and 1.4.4) that gibberellins and cytokinins occur in buds of woody plants and show an increase in their levels at or before the time of bud burst, has led to the suggestion that bud dormancy may be hormonally controlled by an interaction or balance between endogenous inhibitors (ABA) and promoters (GA's, cytokinins and auxins) (Wareing and Saunders, 1971; Bachelard and Wightman, 1974; Nooden and Weber, 1978; Saunders, 1978a; Hanover, 1980).

From this concept of dormancy regulation it is predicted that when the balance between promoters and inhibitors is shifted in favour of inhibitors, dormancy results and growth is resumed after the balance is altered in favour of growth promotion. A shift in balance can be a result of a change in the levels of promoters or inhibitors, or

simultaneous changes in different directions of both promoters and inhibitors. In actively growing shoots of woody species, the presence of growth promoters such as cytokinins and gibberellins have been demonstrated (Zaerr and Lavender, 1980), and therefore, these promoters may be able to counteract and balance the inhibitory effects of the exogenously supplied ABA.

Attempts to shift the balance between promoters and inhibitors, by altering the level of gibberellins present, were made by applying the gibberellin biosynthesis inhibitor, CCC, to the leaves and apical region of actively growing seedlings and AMO 1618, also a GA inhibitor, to shoot tips grown aseptically *in vitro*. No marked inhibition of shoot elongation leading to the cessation of growth and the formation of dormant buds, in whole seedlings or aseptically cultured shoot tips *in vitro*, was observed. The simultaneous application of GA biosynthesis inhibitor and ABA, which was expected to cause a greater shift in the balance between promoters and inhibitors in favour of inhibition, was also unsuccessful in inducing the formation of dormant buds. Although marked inhibition of stem elongation occurred in the presence of ABA when applied alone or in combination with AMO 1618, shoot tips aseptically cultured *in vitro* were still producing new leaves. It appeared ABA was able to slow growth almost to the point that no stem elongation was observable but was not able to induce the formation of dormant buds.

The failure to induce dormancy does not necessarily indicate endogenous ABA has no role in the induction of dormancy as the questions of penetration and metabolism or degradation of CCC and AMO 1618 remain. In the case of whole seedlings, some sites of GA synthesis, such as the roots, may have been unaffected by the exogenously applied CCC, and therefore, the endogenous GA pool remained the same. It was assumed that in the *in vitro* system, using aseptically cultured shoot tips, the penetration of the growth retardant AMO 1618 was not as difficult as in whole seedlings and most sites of GA synthesis were affected. However, in the present study, the aspects of penetration and metabolism of the GA biosynthesis inhibitors were not investigated nor was it established whether GA levels were decreased following CCC or AMO 1618 treatment. It is possible that GA biosynthesis was blocked but the active pool of GA remained the same, in which case there would be no shift in the balance between inhibitors and promoters. Furthermore,

although both CCC and AMO 1618 have been shown to block GA biosynthesis, there is some doubt that inhibition of GA biosynthesis occurs in all tissues and in some cases it has been shown that these growth retardants stimulate the biosynthesis of GA's (Reid and Crozier, 1970; Sembdner, Gross, Liebisch and Schneider, 1980). Nevertheless, both CCC and AMO 1618 act as growth retardants when applied to species of woody plants and are presumed to interfere in the action of GA (Cathey, 1964; Sachs and Hackett, 1972; Nickell, 1978). That the endogenous GA activity in aseptically cultured shoot tips *in vitro* was altered is suggested by the observation that AMO 1618 inhibited, whereas GA_3 stimulated shoot elongation when added to the culture medium separately. However, the stimulatory effect of GA_3 on shoot extension was observed only at the highest GA_3 concentration tested. The presence of GA_3 in the culture medium at lower concentrations was inhibitory to shoot extension. This apparent anomaly in the observed effects of GA_3 on shoot elongation may have resulted from the addition of GA_3 to the culture medium prior to autoclaving. Gibberellic acid has been shown to decompose during autoclaving and the resulting products may or may not have GA-like activity (Van Bragt and Pierik, 1971; Pryce, 1973). The observation that the combined presence of ABA and AMO 1618 in the culture medium resulted in an inhibitory response which was comparable to the presence of ABA alone but greater than in the presence of AMO 1618 alone, suggests that although the promoter inhibitor balance in the shoot tips was slightly affected by AMO 1618, there was no synergistic or additive effect in the presence of ABA. If dormancy was solely a result of the internal balance between ABA and gibberellins, then simultaneous changes in the expected directions of the level of ABA and GA's should have caused the onset of dormancy, or at least some observable additive or synergistic effect on shoot elongation.

Given that the endogenous GA level was altered in such a way as to shift the balance between inhibitors and promoters in favour of inhibtion, then the induction of dormancy might be expected when ABA was applied simultaneously with the GA biosynthesis inhibitors. That dormancy was not induced suggests that a balance between GA's and ABA is not a factor in the regulation of dormancy. However, the failure to induce dormancy does not negate a role for endogenous ABA in this phase of dormancy. The internal balance between promoters and inhibitors during the regulation of dormancy may not only involve GA's but also IAA

and cytokinins. It is possible that the presence of auxins and cytokinins in seedlings and shoot tips was sufficient to counter the inhibitory effect of ABA irrespective of whether the endogenous level of GA was decreased or not. The presence of auxins and cytokinins would ensure the preservation of the balance between promoters and inhibitors.

Besides treatment with ABA or growth retardants CCC and AMO 1618, the removal of the source of supply of growth promoters to and within the shoot would be expected to induce a shift in the balance between inhibitors and promoters in favour of inhibition. The root system of plants has been suggested as a source of growth promoters (Wareing, 1980), especially GA's (Graebe and Ropers, 1978) and cytokinins (Skene, 1975) found in the shoot. Indole-3-acetic acid may also be synthesised in the roots (Schneider and Wrightman, 1978). It was found that removal, by pruning, of part of the root system did not induce bud dormancy but did cause some wilting of the shoot system. The ABA content of plant tissue is known to increase when plants are subjected to water stress (Wright, 1978), and therefore, the inhibitor-promoter balance within the shoot would be expected to be in favour of inhibition as a result of increased inhibitor and decreased promoters. However, as it was not established whether the promoter levels in the shoot were altered after pruning treatment, the failure to induce dormancy is not conclusive evidence that a shift in the promoter-inhibitor balance in favour of inhibition does not result in the onset of dormancy. Auxins (Wrightman, 1973; Schneider and Wightman, 1978) and gibberellins (Graebe and Ropers, 1978) are known to be synthesised in leaves and shoot tips as well as the root system. Therefore, the inhibitor-promoter balance in buds of seedlings may not be easily shifted in favour of inhibition simply by pruning the root system because of the various sites of synthesis of growth promoters.

However, in the *in vitro* system using aseptically cultured shoot tips, it is possible to isolate and manipulate outside sources of growth promotory hormones. The auxin (NAA) and cytokinin (BA) substitutes used in the present study were considered to have been added to the culture medium in minimum amounts required to cause shoot elongation and maintain growth in shoot tips. The absence of NAA from the culture medium resulted in no or little shoot elongation but substantial adventitious bud proliferation, whereas the absence of cytokinin (BA) induced the formation of roots and senescence in the shoots, although some shoot

elongation was observed. It is debatable as to whether the isolated shoot tips are growing as vigorously as possible on the growth regulator supplemented nutrient media or are merely maintaining a much lower but steady level of growth. Their isolation, absence of an exogenous supply of GA's, and a diminishing supply of synthetic auxin and cytokinin may not allow the maximum growth rate and potential to be realised. On the other hand, the supply of water, nutrients and photosynthate (sucrose) could be considered to be optimal. Nevertheless, in the *in vitro* shoot system, it was envisaged that the inhibitor-promoter balance could easily be shifted in favour of inhibition simply by the addition of ABA and/or AMO 1618 to the culture medium. Assuming that a shift in the inhibitor-promoter balance occurred, then the failure to induce dormancy under the experimental treatments suggests an interaction between ABA and the growth promoters is probably not involved in the induction of dormancy. However, evidence that a shift in the balance of inhibitors and promoters had occurred would be required before establishing this as fact. It is possible that for the onset of dormancy, the auxin, cytokinin and gibberellin levels must decline with the simultaneous rise in the level of ABA.

The evidence obtained from the exogenous application studies suggests that endogenous ABA does not have a causal role in the induction of dormancy, and that during autumn the increase in the ABA content of buds of trees growing under natural conditions is not causally related to the increasing intensity of the dormancy state. What evidence, then, is there from exogenous studies with ABA to indicate that the disappearance of endogenous ABA during the winter and spring is connected with the disappearance or emergence of buds from the dormant state?

Several studies (Little and Eidt, 1968; El-Antably et al., 1967; Haissig and King, 1970; Cohen and Kelly, 1974; Powell, 1975b) have tested the ability of ABA to inhibit bud break. The approach in such studies has been to transfer dormant plants, or lengths of shoots with dormant buds intact from dormant trees, into an environment favouring growth, and then record the number of buds which commence growth during a standard period or to record the time taken for bud burst to occur. In the reports just cited, ABA was shown to be effective in reducing the total number of buds that had commenced growth in a standard time, and therefore, suggested ABA can prolong the dormancy of buds.

In the present study and that recently reported by McWha and Langer (1979) ABA was also shown to depress bud burst, but the effectiveness of ABA was modified by several factors. These factors relate to the time of year (that is, dormancy state of the buds), presence and absence of leaves on the shoots, the position of the buds on the stem, and species.

That the dormancy state of the buds changes during autumn and winter was reflected in the number of buds which had commenced growth after their transfer to an environment favouring growth. During the early period of winter, the dormancy state of the buds was most intense with few buds showing a capacity to commence growth. As winter progressed, a less marked dormancy was exhibited by the buds and most buds showed a capacity to commence growth. This observation on the dormancy state of the buds has been reported for many species (see Romberger, 1963; Samish, 1954; Doorenbos, 1953), and the buds during autumn and late winter have been referred to as being in a state of correlative dormancy and imposed dormancy (or quiescence), respectively, whereas during early and mid-winter the buds are in a state of innate or true dormancy (Romberger, 1963; Wareing and Saunders, 1971). Innate dormancy may only last for a short period in winter and the timing between the various dormancy states or phases may vary between species.

Whereas it is accepted that there are various phases in the development of dormancy (Romberger, 1963), there is no agreement on the different states of dormancy which may exist. In the view of dormancy just cited, innate dormancy is considered by some to be a more intense expression of correlative or summer dormancy and quiescence, with the same underlying causes. Others accept Doorenbos's (1953) concept of summer dormancy, winter dormancy and imposed dormancy and consider the first two as being based on distinctly different physiological events. This disagreement complicates any discussion on bud dormancy (e.g. Giertych, 1974), and the use of a variety of terms to describe the different kinds and phases of dormancy has added further confusion. The terms summer dormancy, winter dormancy and imposed dormancy as used by Doorenbos (1953) are equivalent in terminology to correlative dormancy, innate dormancy and quiescence. Some authors refer to summer dormancy or correlative dormancy as quiescence, in recognition that the phases before and after innate dormancy are the same. Others make the distinction that summer dormancy is imposed by correlative influences

(e.g. leaves) within the tree itself, whereas quiescence (in late winter and spring) is imposed by conditions (e.g. temperatures) outside the tree, and therefore, have used the different terms. Horticulturists (Salisbury and Ross, 1978) refer to innate dormancy as rest, and quiescence or imposed dormancy as dormancy. Pre- and post dormancy, and early and after rest, are also terms used to describe correlative dormancy and quiescence or imposed dormancy. Obviously, the removal or clarification of the disagreement and confusion surrounding the description and definition of dormancy would facilitate meaningful discussions on the control mechanisms of dormancy.

Although in the present study no attempt was made to follow closely the changes in the dormancy state of buds of a single species, as winter progressed marked seasonal changes were apparent with a significantly increased proportion of buds growing at the later harvest dates. The qualitative changes in bud growth as recently reported by McWha and Langer (1979) were also observed. These workers reported that the relationship between the position of the buds on the stem and their ability to grow varied not only with harvest date but also with species. In the present study, differences between poplar, willow and alder with respect to the ability of apical and basal buds to grow were apparent. In all species, the position of the bud on the stem was of major importance in determining whether that bud would grow. In willow and alder, apical buds tended to grow, whereas in poplar it was basal buds which tended to grow. Other researchers; Champagnat, Barnola and Lavarenne (1971), Champagnat and Barnola (1975), Meng-Horn, Champagnat, Barnola and Lavarenne (1975), and Crabbé (1980) have also noted differences between the dormancy state of apical and basal buds and, like McWha and Langer (1979), observed that the order of precedence of buds changed with changing seasons. Crabbé (1980) has suggested that this property of buds is of importance in the branching habits of woody plants.

The difference between the response of apical and basal buds cannot be explained solely or partly on the modifying effects of apical dominance especially when the apical buds showed little potential for growth compared with basal buds. Meng-Horn et al. (1975, in a study of *Rhamnus frangula*, suggested that the greatest influence on the bud is a stricly basipetal inhibition, the intensity of which depends on the length of the stem which is in a distal position from the bud. They

also claim the existence of an acropetal stimulation acting only on the terminal bud. Their work seems to indicate that the growth response of the buds situated at lower points on the stem is not an inherent property of the buds but is related more to the length and geometry of the excised shoots. Different plant parts have also been shown (Zieslin and Halevy, 1976) to have an inhibitory influence on the growth of lateral buds of roses where it was found the stem section above a bud had an effect similar to the inhibitory effect of the leaf subtending the bud. The combined initial inhibitory effect of the two parts (stem plus leaf) was found to be more than additive. However, this study on roses was carried out on growing shoots whereas in the studies of Meng-Horn et al. (1975) and the present study, dormant shoots were used. Nevertheless, it is possible that the inhibitory influence of plant parts, that results in correlative inhibition, also has an effect on the development and expression of the dormancy state of lateral buds. Indeed, it has been assumed rather than proven that the dormancy pattern of lateral buds and apical buds is the same, although lateral buds are initially under the influence of correlative inhibition. For apples, Williams, Edwards and Coombe (1979) claim to have verified that the dormancy of lateral buds follows a pattern similar to that proposed previously for buds in general. However, no data as to the number and position of buds on the stem that had commenced growth was presented by Williams et al. (1979), and in view of the present study and that of McWha and Langer (1979) it is possible that qualitative changes in bud growth are observed when bud burst is scored against the position of that bud on the stem.

In the present study with willow, there was evidence that apical dominance had a modifying effect on the relationship between the position of the buds on the stem and their growth. When the inhibitory influence of the apical buds was removed, by decapitating the apical half of the shoots, the basal buds commenced growth. It is possible bud burst was merely a response to tissue injury caused by the cut distal to the buds. Williams et al. (1979) have shown that the dormancy of lateral buds can be overcome simply by decapitation of the shoot or cutting the stem into single bud segments. Therefore, apical dominance may not account wholly for the growth response seen in willow. In poplar, where basal buds showed the greatest tendency for growth, apical dominance cannot account for the response observed.

Abscisic acid was capable of depressing bud burst in all three species used in the present study. However, this inhibitory effect diminished as spring approached. In poplar, where the intensity of bud dormancy was only slight at the time of harvest (spring), the ability of buds to respond to applied ABA was also only slight, whereas in green alder and willow, where dormancy was more marked (autumn and winter harvests), ABA had a greater inhibitory effect. In willow and alder, those buds capable of growth at the various positions on the stem were all affected by the presence of ABA, whereas in poplar only the basal buds were affected by the presence of ABA. Generally, those positions on the stem showing the greatest potential for growth were least affected by ABA, indicating a difference in the sensitivity or ability of buds to respond to ABA. Similarly, McWha and Langer (1979) have noted some indication of a decreasing sensitivity of the apical buds of *Salix alba* and *Salix fragilis* to ABA as spring approached.

Besides bud position on the stem, the presence and absence of leaves also had a modifying effect on the ability of ABA to prolong dormancy. Abscisic acid delayed bud burst significantly in the absence of leaves, although not all the buds responded to the presence of ABA. However, when leaves were present the effect of ABA on delaying bud burst was markedly diminished. It is unlikely that the diminished response was due to less uptake of ABA as it was observed that shoots with attached leaves had increased uptake of ABA solution. In view of the increased uptake, which most probably was a result of leaf transpiration, it was expected ABA would have an increased response. This modifying effect of the presence of leaves on the response of buds to ABA has also been reported by McWha and Langer (1979) who suggested the observation is best explained by the production of a promoter or anti-ABA compound by the leaves. This was supported by the observation, which was confirmed in the present study, of a consistently higher rate of bud burst where leaves were present. However, as noted by McWha and Langer (1979), it is possible that the greater growth recorded in the presence of leaves reflects an increased availability of nutrients from either photosynthesis or redistribution of nutrients from the rapidly senescing leaves. Nevertheless, it appears that the leaves are of major importance in tree dormancy besides the production of some factor which causes dormancy, although some conflicting evidence also exists. For example, Griffin and Robitaille (1979) report that the removal of

leaves from shoots is necessary for bud break to occur in lateral buds of apple shoots, and Janick (1974) reported the removal of leaves after their growth promoter levels had declined, circumvented dormancy in apple trees grown in Java. Similarly, Walser, Walker and Seeley (1981), in a study of peach, claim that if leaves are stripped from peach trees in late summer - early autumn, the dormancy period is reduced in intensity. However, Mielke and Dennis (1975b) found that defoliation did not affect the dormancy of sour cherry flower buds, but did result in the absence of increased ABA levels in the buds. In growing shoots, the leaf has also been shown (Zieslin and Halevy, 1976) to have an inhibitory influence on the bud subtended in its axil. These observations just cited make it difficult to reconcile an apparently promotory influence on bud growth by leaves during dormancy, with an apparently inhibitory influence during shoot growth and induction of bud dormancy.

Irrespective of the modifying effects of the position of the bud on the stem, the presence and absence of leaves and the time of year on bud growth, the failure of ABA to delay the growth of apparently dormant buds after their transfer to an environment favouring growth, may be attributed to other factors such as penetration and metabolism of ABA and the presence of other growth regulators. It is possible, of course, that the metabolism of endogenous ABA and the relative presence of endogenous growth promoters may explain the observance of the modifying effects *per se*. In cases where only the apical buds grew and showed little response to ABA, it is possible that ABA did not reach these buds. However, in the present study, the excised shoots were observed to take up substantial quantities of ABA solution and it is unlikely that uptake or penetration of ABA into the buds was a significant factor in the lack of response of the buds to ABA. If ABA did reach the buds, the possibility remains that ABA was inactivated or compartmentalised within the apical tissues. However, neither penetration or metabolism of the applied ABA was followed in the present study.

There was some evidence to suggest that the presence of growth promoters, particularly GA's, had a modifying effect on the response of bus to the applied ABA. Although the GA biosynthesis inhibitor CCC had little (alder, willow) or no effect (popular) on delaying bud burst, the application of ABA in combination with CCC completely depressed all bud growth or at least resulted in significantly less bud burst than

when ABA (or CCC) was applied alone. Besides the uncertainty surrounding the penetration of CCC and its ability to block GA biosynthesis, the failure of CCC to have a major effect on bud growth, especially in poplar, may be due to the presence of GA's already synthesised in the buds. That GA's are involved in bud break has been suggested on numerous occasions (Wareing, 1969; Wareing and Saunders, 1971; Bachelard and Wightman, 1974) and the observations provide further, although circumstantial, evidence of the involvement of GA's in bud break in an antagonistic role against the presence of ABA. It is possible that the decreasing effectiveness of ABA in delaying bud burst as spring approaches is a result of the increasing presence of gibberellins. However, this hypothesis remains speculative as no attempt was made to determine the endogenous levels of GA's present in the buds, either before or after treatment with ABA and CCC. In addition, CCC was only partly effective and was not comparable to ABA in delaying dormancy. This would not be expected if bud burst is simply a result of increased presence of GA's. Other promoters, e.g. auxins and cytokinins, may also be involved together with the decreasing levels of ABA. It remains to be proven whether endogenous GA's have a causal role in bud break.

It appears from the above discussion that the role of ABA in the maintenance of bud dormancy is not clear or conclusive. Abscisic acid can be shown to delay bud burst but is not totally effective in all species. Furthermore, the response of dormant buds to ABA is modified by effects related to the position of the bud on the stem, the presence and absence of leaves, and the time of year or the dormancy status of the bud. In view of these effects, care must be taken in using the total number of buds that commenced growth as an indication of the dormancy state of the tree and its buds. Therefore, the previous reports in which ABA was shown to prolong the dormancy of buds (El Antably et al., 1967; Little and Eidt, 1968; Haissig and King, 1970) must be reappraised before being accepted as conclusive evidence for a causal role of endogenous ABA in the maintenance of bud dormancy. The evidence, then, from exogenous studies with ABA does not conclusively support the view that the disappearance of endogenous ABA during the winter and spring is connected with the disappearance or emergence of buds from dormancy.

Given that endogenous levels of ABA are not correlated with the onset of dormancy or bud break, and that exogenous applications of ABA to non-dormant and dormant shoots does not result in the induction of dormancy and a dealy in bud burst under some conditions only, respectively, then the simplest conclusion is that ABA does not have a causal role in the regulation of bud dormancy. However, there are some doubts and limitations concerning the methodology and interpretation of results from the two experimental approaches used in the study of the role of ABA in regulating bud dormancy. Where the approach has been to measure endogenous levels of ABA, the failure to demonstrate a correlation between the dormancy state of buds and their endogenous ABA levels does not dismiss a role for ABA in dormancy regulation because a correlation may not be necessary for such a role. A regulatory role may not be based solely on concentration or on pool size. It is possible that the rate of metabolism (biosynthesis and catabolism) of ABA, the concentrations of ABA in specific parts of the bud, tissues and cells (i.e. compartmentation within the cells and tissues of the buds), and the sensitivity of the bud tissues to ABA are more important than the total endogenous level of ABA present. Clearly, further clarification of the activity of ABA within the bud itself is necessary. The evidence from the second experimental approach, the application of solutions of ABA to non-dormant and dormant seedlings and shoots, does not necessarily indicate the role (or a lack of a role) of endogenous ABA as these results may be modified by factors such as penetration and metabolism of the applied ABA or other growth regulators. Only when there is clarification of some aspects of endogenous ABA activity, and the elimination of the doubts and limits of interpretation of the results from the two experimental approaches, can the role of endogenous ABA in the regulation of bud dormancy be conclusively dismissed.

The suggested regulatory role for ABA in bud dormancy is based on investigations on the inhibitor β fraction of plant extracts. The initial studies, in which the inhibitor β content of extracts of leaves and buds was quantified by the use of bioassay methods, demonstrated an apparent variation of inhibitor β with photoperiod and season. The endogenous inhibitor β levels in tree buds during the course of winter showed a progressive decline (Phillips and Wareing, 1958; Kawase, 1966;

Tinklin and Schwabe, 1970) which appeared to be correlated with the gradual loss of dormancy. Similarly, the inhibitor β fraction of buds and leaves was shown to increase in activity following the induction of dormancy by short days (Phillips and Wareing, 1959). Therefore, there appeared to be a good correlation between changes in the inhibitor β fraction and the yearly growth and dormancy cycle of woody plants. Furthermore, the inhibitor β fraction was capable of inducing dormancy in actively growing seedlings (Eagles and Wareing, 1964). Later work isolated and identified the main component of the inhibitor β fraction as abscisic acid (Robinson et al., 1963; Cornforth et al., 1965, 1966; Milborrow, 1967). However, when synthetic ABA and direct chemical methods for assaying ABA became available, a very different picture to that of inhibitor β emerged and sparked the controversy surrounding the role of ABA in dormancy. From the evidence of the present study and that presented by Lenton et al. (1972), it is apparent SD treatment has no effect on the endogenous ABA content of leaves and growing apices. Similarly, the parallel decline in ABA content and dormancy intensity of tree buds is not always close (Mielke and Dennis, 1975b, 1978; Phillips et al., 1979). Furthermore, the application of ABA to actively growing seedlings does not induce the formation of dormant buds. Such contradictory evidence of a regulatory role for ABA in bud dormancy raises two questions concerning the basis for thinking ABA is involved in dormancy regulation: (1) has the inhibitor β fraction, and inhibitors in general, been wrongly implicated in dormancy? and (2) was the inhibitory component of the inhibitor β fraction responsible for the regulation of dormancy wrongly identified as ABA?

The evidence from the present study indicates that the inhibitor β fraction is not correlated with the onset of dormancy. The inhibitory activity of the inhibitor β fraction of apices and leaves of seedlings did not increase after the seedlings were transferred from LD's to dormancy-inducing SD's. Besides the failure to observe an increase in the inhibitory activity of the β fraction, the bioassay data of the present study differed from the data of initial studies (Phillips and Wareing, 1975, 1959) in that little or no auxin activity was detected in the extracts, including extracts of growing apices from seedlings maintained under LD's. It is unlikely in view of the requirement of auxin for growth (Schiedner and Wightman, 1978) that there is a lack of auxin in the tissues extracted. Rather the bioassay data reflects the

relative lack of sensitivity of the wheat coleoptile section test and lettuce hypocotyl test, relative to the oat mesocotyl and coleoptile section tests (Nitsch and Nitsch, 1956). This, together with the assay of each extract at a dilution equivalent to less than 0.25 g dry weight of tissue, may explain the apparent lack or detection of auxin. Furthermore, auxin, or specifically IAA, is known to be very prone to decomposition during extraction and chromatography (Kefford, 1955; Mann and Jaworski, 1970; Iino, Yu and Carr, 1980), and as no special precautions against decomposition were taken it is also possible that losses of auxin occurred during the preparation of extracts for bioassay. Other recent studies with woody plant tissue have also shown little auxin activity on chromatographs of extracts (Alvim et al., 1979; Orchard et al., 1980). Recently, Alvim et al. (1979) has also reported there is no change in the inhibitory activity of the β fraction of willow apices during the photoperiodic induction of dormancy. It is interesting to note that a similar result was observed for *Robinia pseudoacacia* and *Betula pubescens* apices, but not for *Acer pseudoplatanus* in the original study (Phillips and Wareing, 1959) in which it was reported that the inhibitor β fraction of leaves and apices did increase during the photoinduction of dormancy. It appears that the bioassay data from the initial studies on inhibitor β and photoperiodic induction of dormancy are not reproducible. On the other hand, there does appear to be an increase, during autumn, in the inhibitory activity of the β fraction of buds and leaves of trees growing under natural conditions in the field. However, the present inhibitor β investigations suggest the increase is small and occurs after apices have stopped extension growth. This is in agreement with some findings for endogenous ABA where increases in the endogenous content of ABA during autumn (Seeley and Powell, 1981) and the decreases during winter (Mielke and Dennis, 1975b) are not closely correlated with the growth and dormancy cycle. Other studies (given in section 1.4.1) have also shown that there is not a close parallel between the inhibitor β fraction of buds and the growth and dormancy cycle of trees.

It appears there is as much contradictory and controversial evidence concerning inhibitor β levels in buds and leaves and a causal correlation with dormancy as there is for ABA and dormancy. In view of the inability to reproduce the early bioassay data, the simplest conclusion is that inhibitor β is not causally related with dormancy.

However, the study of inhibitor β is complicated by several problems all of which can affect and modify any acceptance of this conclusion or that in favour of the inhibitor hypothesis as reached by the initial investigators. These problems relate to the complex nature of inhibitor β and the limitations of bioassays used to quantify the growth activity of crude extracts containing the inhibitor β fraction.

Besides inherent variation, the use of bioassays for quantification is obviously limited by the degree of sensitivity and specificity of the response of the test objects to a fraction derived from an extract. In the early studies, the tissues or test objects most commonly used to bioassay crude extracts and the inhibitor β fraction of extracts were segments of oat mesocotyl and wheat and oat coleoptiles. These tissues will respond not only to ABA but also to other inhibitory compounds (Wolf, Tilford and Martinze, 1976) which may be present in the inhibitor β fraction. Furthermore, their response to inhibitors may well be reduced by the presence of growth promotory compounds in the β fraction or crude extract. Therefore, interference from substances which co-chromatograph can result in both an overestimate and underestimate of the inhibitory activity of the β fraction depending on the nature of the interfering substance.

That interfering substances, including severl phenolics such as salicyclic and cinnamic acid, can exist in the inhibitor β zone of chromatographed extract is reported by Varga (1957) and Saunders (1978b). Short chain fatty acids have also been claimed to have similar chromatographic properties as components of the inhibitor β fraction (Berrie et al., 1976; Ando and Tsukamoto, 1981), and in the present study C5 and C10 were shown to be inhibitory to coleoptile and hypocotyl growth. Generally, in most bioassays phenolics are inhibitory. However, the inhibitory activity of phenolics varies not only amongst themselves within the same bioassay (Wolf et al., 1976) but also between different bioassays (Turetskaya, Kefeli, Kutacek, Vackova, Tschumakovski and Krupnikova, 1968; Kefeli and Kadyrova, 1971). For example, p-coumaric acid is more active than quercetin in the wheat coleoptile test but both are considerably less effective in the wheat embryo assay. Furthermore, not all phenolics are inhibitory in nature (Thakur, 1977) or active in all bioassays (Kefeli and Kadyrova, 1971). Therefore, it is possible that the inconsistent responses elicited by phenolics in different

bioassays may explain the discrepancies between some of the studies on inhibitor β levels in tissues. Interference in the bioassay due to the presence of known promoters, such as certain GA's located within the inhibitor β zone of chromatographs (Saunders, 1978b), can be most troublesome and be expected to lead to spurious results.

The presence of interfering substances and the failure to separate inhibitors from promoters may account for the pattern or changes in the inhibitor β activity of tree buds observed during the seasonal growth cycle. For example, increased promoter activity co-chromatographing with inhibitors may account for some or all of the decrease in inhibitory activity of extracts of some tree buds as winter progresses. Similarly, an increase in inhibitory activity can be a result of decreased promotory activity. The co-presence of inhibitors and promoters has often been thought of as an explanation for the lack of a close correlation between the dormancy state of buds and their endogenous inhibitor β content. Similarly, it has been suggested (Wareing and Saunders, 1971; Lenton et al., 1972) that interference by contaminating promoters may explain the conflict or difference between the inhibitor β levels and ABA levels of buds observed during the photoperiodic induction of dormancy. This conjecture was supported by the observations that some GA's co-chromatograph with inhibitor β and that GA's can offset the inhibitory effect of inhibitor β, although GA's do not directly promote the growth of coleoptiles which are in their final elongation phase (Thomas et al., 1965). In addition, GA activity has been observed to decrease as dormancy develops (for references, see section 1.4.3). Therefore, the increase in the inhibitor β activity of buds during the photoinduction of dormancy was suggested as being a result of decreased promoter activity (presumably GA-like) within the same (inhibitor β) fraction of extracts while the ABA levels remain the same (Lenton et al., 1972).

Conjecture and speculation concerning the interpretation of bioassay data, obtained in many of the initial studies on the relationship between inhibitor β and bud dormancy, will remain because the term inhibitor β, which was first used by Bennet-Clark and Kefford (1953), does not refer to a specific compound. Rather, by definition, inhibitor β refers to a zone of growth inhibitory activity found on paper chromatographs when an acidic, ether-soluble fraction is prepared from plant tissue extract and occurs at about R_F 0.5 to 0.9 on paper

chromatographs developed in isopropanol-ammonia-water. The definition, then, is based on biological activity together with some general chemical and physical characteristics rather than specific chemical composition. This basis for the definition invites speculation especially with the knowledge that several compounds exist within this zone of the chromatograph, and that ABA accounts for the majority but not all of the inhibitory activity present (Robinson and Wareing, 1964; Milborrow, 1967; Abdel-Bar and Mielke, 1979).

Interference in the bioassay response may be due not only to the presence of natural compounds in the extract but also to the presence of any contaminants arising from the solvents and materials used during extraction of the tissue and fractionation of the extract. Several investigators (Lane and Bailey, 1964; Hartley, Hill, Pegg and Thomas, 1969; Walker, 1971; Winston and Gorham, 1979b; Fraser, 1980) have reported unidentified solvent and chemical impurities that interfere in certain bioassays and can lead to spurious results. Contaminants in solvents have also been reported on numerous occasions to be troublesome in analytical (Martin, Dennis, Gaskin and MacMillan, 1975; Martin and Nishijima, 1977; Iino, Yu and Carr, 1980) and biological (Sharkey and Raschke, 1980) experimentation. In the present study, it was apparent that the growth activity of coleoptiles and hypocotyls was inhibited and promoted, respectively, by the presence of an interaction between the chromatography paper and the solvents used to develop the chromatograph. Whereas Hartley et al. (1969) found the inhibitory activity of blank extracts to be located in several different regions of the chromatograph, Walker (1971), Winston and Gorham (1979b) and Fraser (1980) all found the inhibitory activity of the chromatography system was concentrated at R_F 0.8 to 1.0. The latter observations were confirmed by the present study in which it was found that the wheat bioassay was inhibited and the lettuce hypocotyl bioassay promoted by a chromatography artifact which predictably appeared at the solvent front. Such growth activity is an artifact of the preparatory system and does not reflect the growth response or activity of the extract, and therefore necessitates the use of suitable controls.

The logical choice of controls for bioassays of chromatographed extracts is the bioassay of chromatographs of "blank" extracts, i.e. extracts prepared in the absence of plant material. However, it was found in the present study that there was little or no interference in

the bioassay from solvents used during extraction and purification, and that most of the interference was attributed to the chromatography paper and the solvent mixture used for the development of the chromatographs. That the interfering compound, or compounds, appeared to originate in the paper is suggested by the observation that the degree of interference was not reduced by using redistilled solvents, and that the interference was most intense at the solvent front. Despite this apparent movement of the interfering compound(s) in the developing solvent, the interference could not be removed from the paper by eluting (washing) the paper three times in 80% MeOH. However, the R_F sections of unwashed, undeveloped chromatographs were found to be only slightly inhibitory to coleoptile growth, and therefore, it appeared there was also an effect due to the solvents used for developing the chromatographs. At the solvent front, where interference was most intense, the solvent system presumably concentrated the interfering compound(s) whereas in other R_F sections it appeared the solvent effect was more an interaction with the interfering compound(s) resident in the paper. Since the "blank" extract had no effect on coleoptile growth, and the bioassay response of chromatographs of blank extract and the blank or control chromatographs (i.e. those not loaded with any extract) were similar, it was concluded the pre-methanol washed, solvent run chromatographs would suffice as controls. This choice of control was also used by Fraser (1980).

The necessity of an adequate control for bioassays appears to be obvious. However, it is difficult to ascertain whether adequate controls have been employed in all the initial studies on inhibitor β. To serve as controls, Blommaert (1959) used sections taken from "in front of the starting line of each chromatograph" and Henderschott and Walker (1959b) used a section from "above the original extract band". In both cases, descending chromatograph was used and it is presumed the chromatography sections used as controls are from that part of the chromatograph that was continually submerged in solvent or at least had solvent continually passing through it. Allen (1960) reported using such sections of chromatographs as controls. Similarly, Dennis and Edgerton (1961) used ascending chromatography and took sections "below the point of application of extract" to serve as controls, whereas Wright (1956) reported using "sections taken from the top of the chromatography paper". In many reports, e.g. Phillips and Wareing (1958), Robinson and Wareing

(1964), Ramsay and Martin (1970a), the controls used are not specified, whereas in other reports it is not clear what section of blank chromatographs were used as controls (Hemberg, 1958; Eliasson, 1969) or whether the blank chromatographs used as controls were developed in solvent or not (Hemberg, 1958a,b; Kawase, 1961, 1966). In these studies it is not possible to distinguish naturally occurring growth activity from growth activity in the extraction solvent or chromatography system, and therefore, the significance of any inhibition or promotion found in these studies is open to question.

It appears that the use of blank chromatographs developed in solvent as controls is rare, yet the importance of adequate controls was apparent at the onset of the use of paper chromatography techniques in association with bioassays (Bennet-Clark and Kefford, 1953). It is unfortunate that more recent reports, e.g. Alvim et al. (1979) and Orchard et al. (1980), also fail to indicate the nature of the control used. This practice should be avoided as it encourages speculation on the significance of the promotion or inhibition detected by the bioassays. It is, of course, not known how widespread the problem of solvent and chromatography interference has been (or is) amongst the different laboratories in which studies on inhibitor β were made.

Besides the problem of interfering substances, bioassays are limited by their sensitivity. With bioassays that show a log dose-response curve, e.g. wheat coleoptile section assay, accurate quantitative measurement is limited to the linear portion of the dose-response curve. In addition, such bioassays can be saturated at high concentrations, making it difficult to reveal differences in inhibitor β activity at these saturating concentrations. In the present study, a complete dose-response curve for inhibitor β , when assayed on coleoptiles or hypocotyls, could not be established because of extract overloading on chromatographs. However, the bioassay responses to aliquots of extract indicated that the response to inhibitor β from no response to maximum response was less than two orders of magnitude of extract concentration. In the assay of the inhibitor β fraction, the bioassays were insensitive to low concentrations and became rapidly saturated as the concentration was raised. Assuming no interference from other compounds, it appears that any striking differences between the bioassayed extracts (expressed as histograms) represented only small differences (3 to 5 times) in inhibitor levels. Clearly, the choice of

appropriate concentrations of test extract is critical if valid comparisons are to be made.

The problem of sensitivity is easily circumvented by assaying serial dilutions of the extracts to be compared. Not only will this procedure allow more accurate quantitative measurements to be made but will also detect the presence of natural interfering substances, especially the co-presence of promoters and inhibitors in the same fraction of an extract. This assumes that the concentrations of promoters and inhibitors are sufficiently different to allow their detection and that the bioassay response is distinct. That is, the interaction between the levels of promoters and inhibitors present must be of a nature that allows for either promoters or inhibitors to dominate at one or more of the dilutions assayed. Serial dilutions of the β fraction of balsam fir bark revealed that the β fraction strongly suppressed IAA-induced growth in the *Avena* mesocotyl bioassay except at very low concentrations which acted synergistically with IAA (Clark and Bonga, 1966). In the present study, there was no evidence from the serial dilution of alder extracts for the co-presence of promoters and inhibitors.

Another solution to the problem of interfering substances is to use more than one bioassay in the hope that the activity of the interfering compounds is different in the different bioassays. In the present investigation, two bioassays were employed; the wheat coleoptile section assay and the lettuce hypocotyl assay as modified for inhibitors. Gibberellins occurring within the inhibitor β zone may have interfered with the response of both bioassays to the β fraction. However, this effect would have been minimal as wheat coleoptiles do not respond to GA's when in their final phase of growth, and lettuce hypocotyls are not responsive to GA's in the dark.

In the present study, the inherent variability associated with each bioassay was estimated by the use of many replications and statistical methods (standard errors). Replication was not only at the bioassay level but also at the extract (or sample) and chromatograph level. This ensured that all sources of variation were accounted for in estimating the true experimental error. This is in contrast to the more commonly reported practice of using coleoptiles or hypocotyls treated similarly (e.g. those assayed on the same R_F section) as replicates in the estimation of the experimental error. In this latter case,

replication is at the bioassay level only and any differences between chromatographs are not accounted for. In addition to replication some extracts were assayed at several dilutions, and for all extracts solvent run chromatographs were used as controls in the bioassays. Calibration curves (responses to authentic ABA standards) were used frequently to check the stability of the bioassay response during the assay periods. The high analytical standard was reflected in the agreement found between the two bioassays and between chromatograph and extract replicates within a bioassay. Although problems of specificity (interfering compounds) and sensitivity were not totally overcome, these problems were lessened and determined to the extent that significance of the bioassay data obtained can be judged with some degree of confidence. Therefore, in the present study, the conclusion that the induction of bud dormancy, whether induced naturally during autumn or artificially by photoperiod changes, is not mediated through a rise in the inhibitor β content of buds and leaves appears to be based on sound observation. In view of this conclusion, the role of inhibitor β in the regulation of bud dormancy must be questioned.

It appears that inhibitors from the inhibitor β fraction have been wrongly implicated in dormancy. This then undermines the basis for thinking ABA, the major inhibitory component of the inhibitor β fraction, is involved in the regulation of bud dormancy. However, irrespective of whether inhibitor β is implicated in dormancy or not, the question remains that ABA was incorrectly identified as the major inhibitor responsible for dormancy regulation. Originally, Eagles and Wareing (1964) proposed the term dormin for "substances which appear to function as endogenous dormancy-inducers" following their observation that applications of partially purified inhibitor β fraction, obtained from seedlings maintained under SD's, to leaves of actively growing seedlings maintained under LD's caused the formation of dormant buds. Earlier this group at Aberystwyth had shown there was a rise in the inhibitory activity of the β fraction of leaves and apices of several temperate deciduous trees towards the end of their growing season, and that the photoinduction of dormancy was associated with increased levels of inhibitor β in leaves and apices (Phillips and Wareing, 1958, 1959). However, it was not established whether the same or another component or components of the inhibitor β fraction were responsible for the growth inhibitory activity obtained in the coleoptile section bioassay

and that which appeared to be responsible for the induction of dormancy.

This early work was extended by Robinson et al. (1963, 1964) who attempted to isolate and determine the chemical nature of the growth inhibitory component(s) present in the inhibitor β fraction. They found that the inhibitor β fraction, which was isolated from fresh sycamore leaves, contained several substances, including several phenolics. However, it was found that these phenolics were not the most active inhibitors present, as assessed by the oat mesocotyl assay, and that the main inhibitory activity was thought to be due to a single highly active substance which was non-aromatic. Similarly, Lane and Bailey (1964) found none of the nine phenolics included in their study was identical with the major inhibitor isolated from the inhibitor β fraction of dormant maple buds. Robinson et al. (1963, 1964) were also unable to characterise the inhibitor, but their tests indicated that the inhibitor was an aliphatic or alicyclic compound, possibly a β hydroxy acid. On the other hand, Cornforth et al. (1965, 1966) isolated the sycamore inhibitor, termed "dormin", and identified it as being identical to abscisin II, an abscission accelerating compound that had been isolated in crystalline form from young cotton bolls (Ohkuma, Lyons, Addicott and Smith, 1963) [Abscisin II and dormin were later renamed abscisic acid (Addicott et al. 1968)]. Only a small amount of inhibitor was isolated, and therefore, its effect on dormancy was not tested, although synthetic abscisin II (ABA) was reported to have caused actively growing plants to cease growth and form resting buds when treated daily with ABA. Hence the claim that sycamore dormin was identical with abscisin II (Cornforth et al., 1966). It is, however, interesting to note that Cornforth et al. (1966) extracted their inhibitor from air dried leaves. From the present knowledge of wilting and ABA levels in excised leaves (Wright, 1978), the leaves extracted by Cornforth's group can be presumed to have contained large quantities of ABA, and therefore, it is not unexpected that the major inhibitor in the β fraction as assessed by the excised wheat embryo assay (Miyamoto, Tolbert and Everson, 1961) was found to be abscisin II or ABA. It is possible that the activity of dormin in the bioassay was obscured by the increased levels of ABA present and/or that dormin was not active in the excised embryo assay.

That ABA occurs within the inhibitor β fraction of most plant extracts was shown by Milborrow (1967). Furthermore, he showed that for different plant tissues, the growth inhibitory activity of the β

fraction was almost entirely attributable to the content of ABA. Unfortunately, this evidence has led to the interpretation that inhibitor β and ABA are synonymous, and often original papers on inhibitor β studies are cited in literature reviews and textbooks as studies on ABA. This extrapolation is misleading as the differences in inhibitor β activity do not necessarily reflect differences in the ABA content of similar or same extracts as shown by Alvim, Hewett and Saunders (1976). However, ABA certainly would have contributed to the inhibitory activity of the β fraction in those studies where the fraction bioassayed included ether-soluble acids.

In the present investigation ABA was found to be present in the inhibitor β fraction of alder apices together with phenolic-like compounds. However, these were not as inhibitory to coleoptile or hypocotyl growth as ABA. After the separation, by paper chromatography, of phenolics from the R_F zone containing ABA, the zone corresponding to the R_F of authentic ABA was shown by gas chromatography to contain ABA. No quantification of ABA was attempted, and therefore, it is not known how much of the inhibitory activity of the β fraction can be attributed to ABA, although the ABA zone on chromatographs was markedly more inhibitory to coleoptiles and hypocotyls than zones where phenolics were detected. Furthermore, the increased inhibitory activity in the inhibitor β fraction of apices and leaves during autumn was mainly due to an increase in the inhibitory activity of the zone corresponding to the R_F of ABA, although there was evidence of increases in other unknown inhibitors as well. An increase in the abscisic acid content of apices during autumn has been shown to occur in numerous deciduous trees including apple (Seeley and Powell, 1981) and willow (Alvim et al., 1979). Therefore, it appears from the present study that ABA is the major inhibitor within the inhibitor β fraction and that changes in inhibitory activity of the β fraction reflect the changes in ABA content. However, ABA and inhibitor β are not synonymous as indicated by the comparison of the assays of serial dilutions of extracts with the assays of ABA standards. The dose-response curves were not parallel and only vaguely similar. An increase in the concentration of extract resulted in an increase in the inhibitory activity of most of the R_F sections of the inhibitor β zone, and not just those R_F sections corresponding to the R_F of ABA. However, most of the increase in inhibitory activity was centred around the corresponding ABA sections, and therefore, the

observed response may be due to poor resolution together with overloading.

The responses of the bioassays, used to measure inhibitor β in the present study, also provided further evidence of the presence of more than one inhibitor within the β zone. Although there was close agreement between the bioassays with respect to their response to the β fraction, there were some minor differences. In some cases, the response of the lettuce hypocotyl was relatively greater but confined to a lesser number of R_F sections, whereas the coleoptile response was relatively less but spread over more R_F sections of the same replicate set of chromatographs. It appeared that the bioassays were responding differently, albeit in a minor way, to the presence of inhibitory compounds other than the major inhibitory compound ABA.

The occurrence of other inhibitors besides ABA within the β fraction and the significance of their presence as an explanation for the inconsistency observed between separate studies on inhibitor β levels in buds has already been discussed. However, their presence also poses the question of how relevant to dormancy are the inhibitors detected by the bioassays? A corollary to this question is, how relevant is the use of bioassays based on extension growth responses, to the study of dormancy and/or specifically to the study of dormancy-inducing substances such as dormin? It is unlikely that all the inhibitors detected by extension growth responses in various bioassays are active in dormancy. Conversely, if inhibitors are involved in, for example the photoperiodic induction of dormancy, these need not be inhibitors of coleoptile growth.

The use of the ability to reduce extension growth as a criterion for assessing which is the major inhibitor component within the fraction may be irrelevant to the study of dormancy. From the use of this criterion it appears ABA is the major inhibitor present, but other less active inhibitors are also present and it is possible that one or more of these unidentified inhibitors is of greater significance in dormancy than in extension growth of coleoptiles and hypocotyls. Certainly, if the existence of dormin as demonstrated by Eagles and Wareing (1964) is correct, then it is possible that dormin is one or more of these unidentified inhibitors. It is interesting to note that Hemberg (1949a,b) in his pioneering studies on changes of inhibitory activity in relation to dormancy in buds of *Fraxinus excelsior*, used

the *Avena* curvature test which is not responsive to ABA (Hashimoto and Tamura, 1969b). The identity of these inhibitors remains unknown despite their implication in dormancy.

In relation to dormancy, the possibility that the wrong inhibitor has been identified has arisen from the use of bioassays in which the response parameter has been mostly the inhibition of cell elongation or cell enlargement. These bioassays may not represent dormancy well, although dormancy to some extent does involve inhibition of cell enlargement in the preformed embryonic shoot. However, interfering substances can be troublesome and the usefulness of bioassays based on extension growth responses may be limited to providing primary information on the nature of the endogenous growth substances present in plant extracts. In this respect, the bioassays based on extension growth responses have played an important role in detecting and determining the growth activity properties of the presently known plant growth substances. However, for specific studies on dormancy, it would seem more appropriate to use bioassays which determine the dormancy-inducing properties of the compounds such as the inhibitors (or possibly promoters) detected within the β fraction by, say, the coleoptile assay. Ideally, dormin or dormin-like substances should be assayed by its effectiveness in inducing dormancy in the same species from which the original extract was obtained. However, this is not readily achieved because of technical difficulties associated with the use of tree seedlings as test systems. Although Eagles and Wareing (1964) found successful induction of bud dormancy was possible using seedlings, the induction of dormant buds is too imprecise an effect to be used quantitatively. A more convenient quantitative bioassay is required.

Theoretically, aseptically cultured shoot tips *in vitro* could substitute for whole tree seedlings, and therefore, provide the basis for a novel bioassay technique for the detection of dormin-like substances. It should be remembered, however, that an *in vitro* system may not parallel the intact system and that observations such as the successful induction of dormancy *in vitro* may have little significance in the intact plant. Final proof of the presence of dormin or dormin-like substances rests with its dormancy-inducing properties as determined on whole seedlings. In the present study, attempts were made to induce dormancy in growing shoot tips cultured *in vitro*, and whilst

the photoperiodic induction of dormancy was possible the use of shoot tips as test objects in a quantitative bioassay was limited by two major factors.

First, the growth of individual shoots was highly variable, both quantitatively and qualitatively. Ideally, a single growing shoot was required throughout the culture period, but more often than not multiple shoots developed from the apparently single shoot tips, and in some cases, callus formation together with adventitious bud proliferation occurred at the shoot bases. The selection of apparently single shoot tips with the least amount of callus and bud proliferation before use, reduced but did not eliminate the problem of variable qualitative growth. The growth of visually comparable single shoots was also variable over the culture period. The elimination of qualitative and quantitative variation should be possible, either by the manipulation of the medium composition or through the use of less variable species or clones.

The second limitation to the use of shoot tips grown *in vitro* is related to the assessment of dormancy; what criteria should be used to judge a bud as being dormant? One needs to distinguish between the simple cessation of growth and innate dormancy. This problem of the precise defintion of dormancy is, of course, not limited to *in vitro* studies but to all studies related to tree dormancy. Saunders (1978a) has defined a dormant bud "as one which is not subject to the inhibitory effects of apical dominance but in which growth is nevertheless restricted to such an extent that bud burst is indefinitely delayed, even when the plant bearing the bud is maintained under environmental conditions of temperature, nutrients and water supply, which would normally permit shoot extension." Others, Samish (1954), Doorenbos (1953) and Romberger (1963), have also defined dormancy similarly, although as discussed earlier, there is little agreement between the terms used to differentiate between the various phases in the development of dormancy.

For experimental purposes, the degree of dormancy possessed by tree buds is assessed by the time required to commence growth, or the degree of chilling (i.e. the number of hours at low temperatures) required before buds commence grwoth under standardised conditions which are favourable for growth. It is also possible to use these tests

in the *in vitro* system. Hence, the growth response of apparently dormant shoots, after their transfer from a dormancy-inducing medium to a medium that normally supports growth, can be used to assess their dormancy state by determining the time required for growth to recommence, or the degree of chilling (or alternatively the amount of GA) required to break dormancy. However, this may be an oversimplification of the dormancy state. For example, buds may not be dormant yet growth will not recommence because of the immediate medium composition, which may normally be favourable for continued growth but not favourable for the initial phase of growth. A vitamin or enzyme cofactor may be lacking or in excess. Therefore, precise media composition would need to be clarified. Furthermore, this method of assessment of dormancy is time consuming.

Other than the simple qualitative response of growth or no growth, simple quantitative growth responses such as change in fresh weight, dry weight, and increase in stem elongation can be used. However, these responses are also an oversimplification of dormancy and, of course, require a destructive harvest technique for their determination.

The most convenient indicator of the dormancy state would be a visual marker, as the definition of dormancy describes the morphological symptoms of a physiological state, e.g. the reddening of bud scales is characteristic of the formation of dormant buds in some alder species. However, the rapid micropropagation *in vitro* of *Alnus* species is difficult as shown in the present study. It is unlikely that other morphological features of dormant buds could be visible to the naked eye. On the other hand, some morphological and anatomical features will be visible under the light microscope and these could be used as dormancy indicators. For example, in a study (Goffinet and Larson, 1981; Larson and Goffinet, 1981) of cottonwood (*Populus deltoides*) during dormancy induction, distinct structural changes within the buds and in the vascular transition zone occurred. Within the bud, lamina of the bud-scale leaves at LPI-1 and LPI-2 aborted, whereas the stipules at these positions developed into bud scales following dormancy induction. Consequently, the ratio, total leaf length to stipule length, decreased rapidly. This ratio could be of use in the rapid assessment of dormancy in this species. Some histological and cytological events that accompany the seasonal cycle of tree bud growth could also be used to estimate dormancy. However, there are few

ultrastructure studies on buds of woody species. Some recent studies (Bagni, Marino, Torrigiani and Andisio, 1977; Cottignies, 1977, 1979; Alsaidi and Bouard, 1979; Willison and Cragg, 1980; Cragg and Willison, 1980; Lynch and Rivera, 1981) indicate that there may be some differences between dormant and non-dormant cells that may be useful indicators of the dormant state, such as changes in the nucleolar size and activity, nuclear pore sizes, ribosomes, and proplastids containing lipids and starch. However, the generality between species of some of the ultrastructural features observed is yet to be determined. Other cell studies (Owens and Molder, 1973; Carlson, Binder, Feenan and Preisig, 1980) indicate that mitotic frequency (the number of dividing cells in five median longitudinal sections) and mitotic index (the percentage of cells in division) can also be used to monitor the onset of dormancy, with the latter method being more precise and rapid than the former.

Physical methods may also prove to be useful in evaluating the dormancy status of buds. Of particular interest is the measurement of CO_2 exchange in growing and dormant shoots. It appears that there is a seasonal pattern of CO_2 exchange in the shoots of some woody species (Bachelard and Wightman, 1973; Drew and Ledig, 1981) with photosynthetic and dark respiration rates declining rapidly during dormancy. The *in vitro* system with aseptically cultured shoots is technically amenable to CO_2 exchange studies, and therefore could be useful.

A physical method that has been used to evaluate dormancy in the oscilloscope technique proposed by Ferguson, Ryker and Ballard (1975). An oscilloscope and square wave generator were used to measure the electrical resistance to square wave pulses through a portion of the shoot. However, it appears that this method is of little value because of difficulties in interpretation and obtaining reproducible quantitative data (Timmins, Fuchigami and Timmins, 1981; Holbo, Askren and Hermann, 1981).

The apparent lack of suitable indicators of bud dormancy in woody plants and the inherent variability, limits the use of the *in vitro* shoot system as a quantitative bioassay. Nonetheless, the system can still be used qualitatively to detect the presence of dormin-like substances. In the present investigation, no such substances were detected by the *in vitro* shoot system in either the acidic ether-soluble fraction, including the inhibitor β fraction of apices and leaves, or the aqueous fraction of an 80% MeOH extract of dormant shoot tissue.

It was expected that, if dormin-like substances exist, they would be present in leaf and bud tissue of seedlings which had been naturally or artificially induced into dormancy. The failure of detection does not necessarily imply that dormin-like substances are absent in dormant tissues. Substances such as the hypothetical dormin may have been present in the basal medium containing the test fraction of the extract, but their effect on shoot growth may have been modified by the lack of penetration into the tissue, or after penetration and uptake, by an inactivation mechanism present in the tissue. Furthermore, the presence of synthetic auxin (NAA) and cytokinin (BA) may have offset the activity of the dormin(s). In the crude extract (aqueous fraction of 80% MeOH extract), naturally occurring promoters including cytokinins, auxins and gibberellins would also have been present. The extent and influence of these were not determined, but the crude extract was generally inhibitory to shoot growth. Whether all such extracts, including extracts of growing shoot tissue, are inhibitory was not tested. It is also possible that the range of serial dilutions of extract assayed was not sufficient to allow the detection of dormin(s) because the level of dormin(s) present was too low to permit detection. Dormin(s), of course, may have been absent because of degradation during autoclaving.

The stability of such compounds under *in vitro* conditions is unknown, as is their chemistry and identity. If the existence of dormin, as demonstrated by Eagles and Wareing (1964), can be repeated on whole seedlings, then its detection and assay by the *in vitro* shoot tip system should be possible. In the final analysis, the significance of any observation made *in vitro* must be supplemented with intact studies, and vice versa if meaningful conclusions are to be made. In this respect, failure to demonstrate the existence of the hypothetical dormin(s) was not absolute.

The apparent lack of a causal correlation between the endogenous inhibitor β levels of buds and leaves, and dormancy, and its apparent failure to induce dormancy suggests that inhibitor β and possibly inhibitors in general have been wrongly implicated in dormancy. This, together with the inconsistent evidence in favour of a role for ABA in dormancy, suggests some other substances similar to the hypothetical dormin could be involved in the regulation of bud dormancy.

Before the discovery of abscisic acid, phenolics, including those that occur within the inhibitor β fraction, were thought to play an

important role in the control of dormancy (Hemberg, 1961; Kefeli and Kadyrova, 1971). Several phenolics, e.g. catechin, caffeic acid, cinnamic acid, coumarin, o- and p-coumaric acid, ferulic acid, salicyclic acid, kaempferol, quercetin and naringenin, have been identified as occurring in buds of woody species, including sour cherry (Szember and Wocior, 1976a,b), silver maple (Lane and Bailey, 1964), peach (Altree-Williams et al., 1975) and sugar maple (Thakur, 1977). In the present study phenolics were tentatively characterised as occurring in alder buds.

A correlation between the seasonal growth activity and endogenous levels of specific phenolics has been found in buds of sour cherry (Szember and Wocior, 1976a,b) and sugar maple (Thakur, 1977). However, as yet it has not been established whether or not phenolics possess dormancy-inducing properties. Furthermore, it is not clear whether endogenous phenolics are acting as inhibitors of growth (Ray, Guruprasad and Laloraya, 1980). Recently, Ray et al. (1980) found that phenolic compounds including trans cinnamic acid, ferulic acid, coumarin and naringenin can antagonise the inhibitory action of exogenous ABA on hypocotyl growth of light grown *Amaranthus candatus* seedlings. It appears in this case, phenolics were acting as promoters, although it was not established whether the phenolics tested were promotory or inhibitory to hypocotyl growth in the absence of ABA. Tantvydas (1979) found that the phenolic compound phlorizin (phloretin-2'-β-D-glucoside) enhanced the GA_3-stimulated elongation of lettuce hypocotyls. Without GA_3, phlorizin had no effect on hypocotyl growth in the light but it inhibited growth in the dark. Similarly, Jones (1976) has shown that phloridzin promotes the growth of cultured apple shoots. Therefore, the growth properties of the endogenous phenolics may be promotory or inhibitory. Furthermore, the extent and level of phenolics in buds of different species seem to vary considerably. Some phenolics appear to occur in much greater quantities in extracts of certain species only. For example, phloridzin in apple is unique to the species *Malus*, salicyclic acid in willow, and naringenin in peach. Their occurrence is more likely related to the specific metabolism peculiar to that species rather than being of physiological significance in the regulation of dormancy.

It appears that short chain fatty acids may be involved in the dormancy of bulbous plants (Tsukamoto, Ando and Yazawa, 1973; Ando and

Tsukamoto, 1974) and wild oat seeds (Berrie, Don, Buller, Alam and Parker, 1975). It has been reported that endogenous short chain fatty acids are present in high concentrations in various plant tissues, including the buds of woody species (Berrie et al., 1975), and that they have similar chromatographic properties as the inhibitor β fraction (Berrie et al., 1975; Ando and Tsukamoto, 1981). The response of some plant tissues to short chain fatty acids is similar to their response to ABA, e.g. stomatal closure in epidermal tissue (Wilmer, Don and Parker, 1978), GA induced amylolysis in the barley aleurone layer (Buller, Parker and Reid, 1976), germination of mustard and lettuce seeds (Le Poidevin, 1965; Stewart and Berrie, 1979), and growth of wheat coleoptiles (Ando and Tsukamoto, 1981). These tissues respond to short chain fatty acids in a manner similar to ABA in that acids appear to be functioning as inhibitors. The short chain fatty acids, however, do differ from ABA in that their dose-response curves are not logarithmic but exhibit an arithmetic effect, with the change from no effect to maximum effect occurring over a very small concentration range (Berrie et al., 1976). Furtheremore, at high doses the fatty acids are toxic to plant tissues.

In the present study, short chain fatty acids were found to be inhibitory to wheat coleoptile and lettuce hypocotyl growth. However, the inhibitory power of a short chain fatty acid appears to differ between the two bioassays. This may be related to the differences between excised (coleoptile sections) and intact (lettuce hypocotyls) tissues. At the high concentrations (greater than 10^{-3}M), short chain fatty acids appear to be toxic to plant tissues, especially meristematic and young differentiating tissues. However, all tissues are likely to be affected as the short chain fatty acids appear to affect the lipid bilayers of membrances such that proton permeability is increased and the fluidity of membranes changed (A.M. Berrie, pers. comm.). This property of toxicity has enabled derivatives of short chain fatty acids to be used as commercial chemical pruning agents in the control of lateral bud growth (Cathey and Steffens, 1968; Cathey, Steffens, Stuart and Zimmerman, 1966; Steffens, Tso and Spaulding, 1967; Maw, 1977; Tucker and Maw, 1975; Tso, 1964). Selectivity of tissue damage or kill is achieved through the use of specific surfactants such that terminal buds only, or lateral buds only, are killed. The killing action of the short chain fatty acids was effective only when the buds came in direct

contact with the meristematic tissue, and the chemicals did not appear to be translocated or metabolised by the plant tissue (Tso, Burk and Steffens, 1966).

In the present study the applications of short chain fatty acids C5, C8, C9 and C10 to growing seedlings, and C5 and C10 to aseptically growing shoot tips *in vitro*, failed to induce the formation of dormant buds. It appears short chain fatty acids do not have dormancy-inducing properties. Furthermore, their effect on stem growth of aseptically cultured shoot tips *in vitro*, and on bud burst of apparently dormant buds on isolated shoots, suggests that they can be promotory as well as inhibitory. This dual feature of potential growth activity has also been noted for other fatty acids including palmitic, stearic, oleic, linolenic, and linoleic when applied to wheat coleoptile sections in the presenceof ABA (Mielke and Flower, 1979). The failure of short chain fatty acids to be inhibitory and/or induce dormancy may be related to the problem of the low solubility of short chain fatty acids in water and to their toxic effect on tissue.

Short chain fatty acids of chain lengths greater than eight carbons are only sparingly soluble in water at room temperature, and this may account for their lessened activity and the need for high concentrations to elicit any response from the tissues. Similarly, where toxicity occurred, the amount of short chain fatty acids reaching the active sites would be limited because of the apparent lack of translocation from the toxic zones. On the other hand, growth inhibition may be merely a result of a physical effect of short chain fatty acids on membranes, rather than a physiological effect on membranes and cell organelles or cell metabolism in general. Indeed, the very rapid change in response to concentration gradients, and when the maximally effective concentration is reached, the short jump to toxicity, all appear to offer little in the way of a control system. Presently, it would be premature to speculate on possible means by which endogenous levels of short chain fatty acids are regulated to avoid toxic reactions and yet remain regulatory in growth processes. However, the possibilities of compartmentation and conjugation are obvious. The apparent promotory effect of short chain fatty acids is difficult to reconcile with their observed inhibitory effects. Perhaps the dual feature of growth activity is merely a result of the non-specific effect of short chain fatty acids on membranes. Whatever the mechanism, the results from the present study

provide no evidence of a role for short chain fatty acids in the control of dormancy.

It appears the search for the hypothetical dormin will need to continue beyond the presently known substances that are active in growth. Indeed, whilst the inhibitor β and ABA notions of the inhibitor hypothesis appear to be wrong, the basic concept of a messenger involved in dormancy remains. The evidence for the inhibitory influence of SD-treated leaves upon the growth of the apex in woody plants is most convincing (Wareing, 1954; Waxman, 1957). That daylength perception resides in the leaves of some woody plants, whilst the growth reaction resides at the apex, suggests the involvement of a messenger. The messenger has been demonstrated to be inhibitory in nature, as the cessation of growth induced by the exposure of leaves to SD's cannot be simulated by simply removing the leaves. If the messenger was a growth promoting factor, which was synthesised under LD's and was essential for growth, defoliation would substitute for SD's. A messenger, whether chemical or physical, must be able to substitute for the SD influence on shoot growth. The identity of this messenger could provide the key to the control mechanism(s) involved in the regulation of bud dormancy. The present study suggests that this messenger is not ABA or any of the inhibitors found within the inhibitor β fraction of plant extracts.

What role, then, does ABA have in the growth and development of woody plants. It appears to be ubiquitous within the woody plant and amongst woody species. A role for ABA as an endogenous inhibitor, either *per se* or by counteracting the effects of growth promotory substances, is not consistent with its distribution and levels within the plant. For example, young leaves expand rapidly despite having a very high ABA content. As an explanation, Powell (1978) has suggested that ABA is merely acting as a brake on growth; that in the absence of any ABA the young leaves and shoots would expand more rapidly than they normally do. This idea is yet to be tested. Amongst the more recently accepted roles for ABA (Zeevaart, 1979; Walton, 1980), a role in water stress through the regulation of stomatal aperture function, has received the most attention. Other roles such as root geogropism, seed and fruit development and maturation, and sugar and mineral ion transport are also being actively researched.

A role in the development of cold hardiness (the ability to

withstand low and freezing temperatures) and protection from chilling injury has also been suggested and received some attention (see Holubowicz, 1978; Rikin, Atsmon and Gitler, 1979; Levitt, 1980). Cold hardening in woody species normally begins in the autumn under the influence of SD's and low temperatures (Weiser, 1970; Young, 1970), and there appears to be a relationship between the seasonal changes in sugars and starch content of tissues of woody species, and cold hardiness (Jones and Steinacker, 1951; Dowler and King, 1966; Young, 1969; Lasheen, Chaplin and Harmon, 1970; Lasheen and Chaplin, 1971; Nelson and Dickson, 1980; Kaurin, Junttila and Hansen, 1981). Sucrose is known for its cryoprotection action (Sakai and Yoshida, 1968; Dear, 1973; Seibert, 1976; Levitt, 1980), and reducing sugars have also been implicated (Sieckmann and Boe, 1978; Purvis, Kawada and Grierson, 1979). That ABA could be involved in the hardening process is suggested by the observations of Holubowicz and Boe (1969) that ABA increased the hardiness of apple seedlings, and of Chen, Gavinlertvatana and Li (1979) that the frost hardiness of *Solanum tuberosum* leaf tissue was increased by the presence of ABA. Chen et al. (1979) also found that ABA was capable of substituting for low temperatures in inducing frost hardiness in *Solanum commersonii*. Furthermore, there is some evidence to suggest ABA is involved in sugar metabolism and transport (Karmoker and Van Steveninck, 1979; Tanner, 1980). Therefore, it is possible that ABA could have a role in cold hardiness by controlling the carbohydrate metabolism such that the protective presence of sugars is maintained during the winter.

In the present study, the effect of ABA on the accumulation of sugars and starch in aseptically grown shoot tips *in vitro* was investigated. The results were inconclusive; a consistently higher but non-significant increase in total soluble sugars was found in the ABA treated shoots compared to untreated shoots. Perhaps a more positive correlation exists between reducing sugars and ABA as opposed to total soluble sugars. Karmoker and Van Steveninck (1979) found ABA caused an increase in the content of reducing sugars more than total soluble sugars in roots and stems of bean plants. Furthermore, Purvis, Kawada and Grierson (1979) found reducing sugars were better correlated with resistance to chilling injury than total soluble sugars in grapefruit peel. However, a relationship between ABA, reducing sugars, and cold hardiness is yet to be proved.

Whatever the role of ABA in woody species, it may be productive to remember that a role may not be determined on the basis of correlation studies.

CHAPTER 5

CONCLUSIONS

It appears that growth inhibitors including ABA, short chain fatty acids and phenolics have been wrongly implicated in bud dormancy. The initial bioassay data on which the inhibitor hypothesis of dormancy was based does not appear to be reproducible. Inhibitor β levels in buds and leaves are not causally related to shoot growth and bud dormancy. The present evidence is not consistent with the inhibitor hypothesis of dormancy or the inhibitor-promoter balance concept of dormancy regulation.

The evidence from the present studies with ABA does not support a dominating role for ABA in the regulation of dormancy. High levels of ABA present in young growing apices raises doubts as to the role of ABA as an inhibitor of shoot growth. Seasonal changes in the ABA levels of tree buds do occur, but these are not causally related to the regulation of dormancy. It appears that correlations of ABA content with bud and shoot growth are misleading as the photoperiodic induction of bud dormancy does not involve a change in the levels of ABA present in the buds. Furthermore, exogenous applications of ABA to actively growing seedlings does not result in the induction of dormancy. The ability of ABA to prolong the dormancy of apparently dormant buds is modified by several factors, and a delay in bud burst occurs under some conditions only.

The simplest conclusion from the evidence is that ABA does not have a causal role in the regulation of dormancy. However, the past and present investigative approaches used in the study of dormancy regulation may obscure the role of ABA. Little is known of the cellular compartmentation of ABA, or changes in its rates of biosynthesis and catabolism or the nature of inactivation mechanisms. Similarly, the rates of transport to active sites and whether the sensitivity of apical tissue to ABA changes during the course of dormancy are unknown. These aspects of ABA activity within the bud deserve some attention.

The presently recognised hormones may all be involved in the regulation of bud dormancy. However, little is known of the relationship between the hormones during the progress of dormancy, and it appears from the present study that an interaction between ABA and GA's is not involved in the regulation of dormancy. There is little evidence to suggest auxins and cytokinins interact with ABA to control dormancy.

The key to our understanding of the hormonal regulation of dormancy is dependent on the existence of a dormancy-inducing substance(s) such as the hypothetical dormin. The present evidence indicates that dormin is not ABA and possibly not a component of the inhibitor β fraction of plant extracts. The development of a suitable bioassay for dormin-like substance(s) is imperative and the use of aseptic shoot tips cultured *in vitro* for this purpose appears promising.

Further understanding of the hormonal regulation of dormancy may be obtained from studies on the histological, cytological and biochemical events accompanying the seasonal cycle of growth in woody plants. A correlation may exist between hormonal changes and some of these events although a correlation will not necessarily provide an indication of a causal relationship. The study of these events may also assist in defining the various phases of dormancy.

Since changes in inhibitor or promoter concentrations or a simultaneous change in the concentration of both fails to explain the regulation of dormancy, the hormonal concept of growth substances may need to be questioned.

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Mrs A.J. Dellow typed the thesis.

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